UNIFORMED SERVICES UNIVERSITY, SCHOOL OF MEDICINE GRADUATE PROGRAMS
Graduate Education Office (A 1045), 4301 Jones Bridge Road, Bethesda, MD 20814

FINAL EXAMINATION/PRIVATE DEFENSE FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN THE MOLECULAR AND CELL BIOLOGY GRADUATE PROGRAM

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Date of Examination: August 22, 2012

Time: 2:00pm

Place: A2054

DECISION OF EXAMINATION COMMITTEE MEMBERS:

	PASS	FAIL
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DISSERTATION APPROVAL FOR THE DOCTORAL DISSERTATION IN THE MOLECULAR AND CELL BIOLOGY GRADUATE PROGRAM

Title of Dissertation: "Identification of a novel Bcl10 domain that contributes to NF-κB activation"

Name of Candidate: Ashley C. Shaloo
Doctor of Philosophy Degree
August 22, 2012

DISSERTATION AND ABSTRACT APPROVED:

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Abstract

The NF-κB transcription factor is a centrally important mediator of T cell functional responses. However, much is still unknown of the regulation of this pathway. Activation of the NF-κB signaling cascade via numerous cell receptors, including the T cell receptor (TCR), requires the adaptor protein Bcl10. The function of Bcl10 in the NF-κB pathway, as well as other pathways, is not yet completely understood. In this work, I investigated the function of a 12-amino acid peptide on the Bcl10 N-terminus, dubbed the N-terminal peptide. As there is currently no crystal structure of the Bcl10 protein, I developed N-terminal peptide mutants, targeting amino acids that are potentially important to the function of the peptide. Through mutation of key amino acids to alanine as well as deletion of groups of amino acids, I determined that the N-terminal peptide functions to positively influence Bcl10 activation of downstream NF-κB. This research also supports the function of the N-terminal peptide in Bcl10 binding to partner proteins, including CARMA1 and MALT1, as well as Bcl10 intracellular redistribution, and Bcl10 protein stability. These data establish the N-terminal peptide of Bcl10 as a novel determinant of Bcl10 function. Mutation or deletion of the N-terminal peptide causes Bcl10 hyperphosphorylation, which then leads to premature Bcl10 cleavage and degradation. Based on these findings, I propose that TCR activation leads to PKC-mediated phosphorylation of Bcl10, targeting hyperphosphorylated Bcl10 for cleavage by an unknown protease, followed by rapid ubiquitination and subsequent degradation by the proteasome. This Bcl10 degradation process represents a negative feedback mechanism to limit NF-κB activation by the TCR. Upon mutation of the N-terminal peptide, Bcl10 becomes predisposed to spontaneous phosphorylation by PKC, resulting in accelerated steady-state degradation. This rapid turn-over, combined with a reduced ability to interact with key binding partners, causes N-terminal peptide mutants to exhibit impaired NF-κB activation. I hypothesize that the N-terminal peptide functions to stabilize the Bcl10 protein in the steady-state, and to directly or indirectly contribute to protein-protein interactions with a subset of signaling partners. This data suggest that the Bcl10 N-terminal peptide represents a good candidate for therapeutic modulation of specific Bcl10 functions in diseases of dysregulated NF-κB activation.

Identification of a novel Bcl10 domain that contributes to NF-κB activation

By

Ashley Cora Shaloo

Dissertation submitted to the Faculty of the Molecular and Cell Biology program

Uniformed Services University of the Health Sciences

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy, 2012

Acknowledgements

My gratitude to my thesis advisor, Dr. Brian Schaefer, for his mentorship and patience over the past few years.

Much appreciation to my thesis committee members: Dr. Edward Mitre, Dr. Gabriela Dveksler, Dr. Johnan Kaleeba, and Dr. Paul Love, for their constructive criticism and guidance.

Thanks to members of the Schaefer lab that I have known through the years (Sean, Anuj, Natalia, Mouna, Joe, Michael, Suman, and Lara).

Thank you to Dr. Louise Wootton for sparking my initial endeavors into biological research.

My support crew and family I could never thank enough, and do here resoundingly thank for their support, encouragement, laughs, and love (Dad, Mom, Megan, Anna, Grandma, and the Vinci and Shaloo clans).

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Introduction

T cells and the immune response

The immune system is composed of two branches, the innate and the adaptive immune response [1]. The innate immune response is characterized by its direct response to pathogens in an immediate and conserved manner. Cells of the innate immune response are directly responsible for inflammation and include neutrophils, natural killer cells, macrophages, dendritic cells, mast cells, and basophils. Although there are more subgroups of innate immune system cells, there are several major groups including phagocytic cells (e.g. macrophages, neutrophils), which use specific receptors to identify foreign antigen and ingest it for the purpose of antigenic processing and pathogen clearance; granulocytes (e.g. neutrophils, basophils), which release endocytic vesicles containing chemicals such as hydrogen peroxide and histamine to damage pathogens; and cytotoxic lymphocytes (e.g. natural killer cells), which release apoptosis-inducing cytokines directed at host tissue that is expressing aberrant cell-surface markers indicative of infection or mutation. The innate immune response also utilizes a humoral mechanism called the complement system. Complement activation plays several roles, including recruiting innate immune system cells such as neutrophils and macrophages to facilitate pathogen destruction. Complement also aids in the adaptive immune response by coating antigen-antibody complexes, which facilitates removal by phagocytic cells. Although the innate immune response is rapid, responding on the order of hours and days, it is the more slowly activated adaptive immune response that clears more persistent and recurring pathogens.

The adaptive immune response offers the host a protective immunity from pathogens by developing antigenic-specific memory cells. The adaptive immune system is characterized by its highly specialized cells, which are clonally expanded through exposure to pathogen antigens. One method by which the adaptive immune response is initiated is when phagocytic cells such as dendritic cells, macrophages, and B cells, also known collectively as antigen presenting cells (APCs), ingest foreign pathogens. The APC then breaks down the pathogen into its component antigenic peptides, which are then coupled with a protein of the major histocompatability complex (MHC) and presented on the cell surface. Presented in conjunction with the MHC, the pathogenic antigen can then be recognized by another type of adaptive immune system cell known as T lymphocytes (T cells; Figure Intro-1).

There are two types of MHC molecules, referred to as class I and class II. The exogenous pathway of pathogen processing involves the phagocytic engulfment of a pathogen (e.g. by B cells) and the presentation of pathogenic antigen on the B cell surface by the MHC type II receptor. This exogenous pathway can be utilized for nearly any type of extracellular pathogen that can be phagocytosed either in whole or part, including bacteria and parasites. The endogenous pathway of antigen processing occurs when any nucleated host cell is infected by an intracellular pathogen (such as a virus), and the cell presents antigen on its cell surface in the context of MHC type I receptor.

The MHC bound to peptide is recognized by a T cell antigen receptor (TCR), which is found on the surface of two main subgroups of T cells known as $CD4^+$ and $CD8^+$ T cells. These two T cell subgroups along with all T and B cells develop into memory cells that recognize a singular and specific antigenic epitope through a strict antigenic presentation selection process [2]. This selection process induces genetic recombination of the cell's receptor (e.g. T cell

receptor) to one that will only recognize the unique antigen of interest. Although each T cell only recognizes one specific antigen, taken together, all of the T cells within an organism represent a vast index of antigen receptors. With this vast index a host experiencing a secondary infection by a pathogen it has already been exposed to will elicit a much quicker and specific immune response due to the antigen-specificity of the memory T and B cells developed during the pathogen's initial infection.

As mentioned earlier, the immune system has two pathways of antigenic processing: exogenous and endogenous. Each pathway offers one option of response by the adaptive immune system's memory T cells. For virus-infected host cells of the endogenous pathway, $CD8^+$ T cells recognize and bind through their T cell receptor (TCR) to pathogen antigen presented by MHC type I (Figure Intro-1). The response elicited by CD8+ T cells bound to its specific-antigen is analogous to natural killer cells in that they directionally release endocytic chemicals that destroy pathogen-infected host cells. The exogenous pathway of antigen processing involves the MHC type II, which is found only on APCs and elicits the response of $CD4^+$ T cells, also known as "helper" T cells. These helper T cells bind to antigen presented by an APC through their TCR and accordingly begin a program of cellular differentiation that helps the cell to better respond to infection by releasing cytokines that stimulate growth and development of effector functions in an autocrine manner. Helper T cells also 'help' neighboring immune cells by releasing cytokines that act in a paracrine manner to recruit and stimulate other immune cells (e.g. B cells) into developing effector functions to better fight infection.

T cells rely on multiple receptors in addition to the TCR to recognize antigen, including either CD4 (recognizing MHC-II antigen) or CD8 (recognizing MHC-I antigen), and if the T cell

is binding to antigen for the first time (naïve), the CD28 "co-stimulation" receptor also plays a crucial role (Figure Intro-2) [3, 4]. This method of co-stimulation is concerned with a second subset of intracellular signaling intermediates that need to be supplemented by a receptor (e.g. CD28) in addition to the TCR to initiate downstream signaling of cell development (Figure Intro-3). This method of receptor co-stimulation is a safeguard against inappropriate responses to self-antigen (autoimmunity). Without this concurrent co-stimulation in naïve T cells, the cell wouldn't receive the downstream intracellular signals at a strength necessary to promote cell differentiation. T cell anergy would result, which is a form of peripheral tolerance.

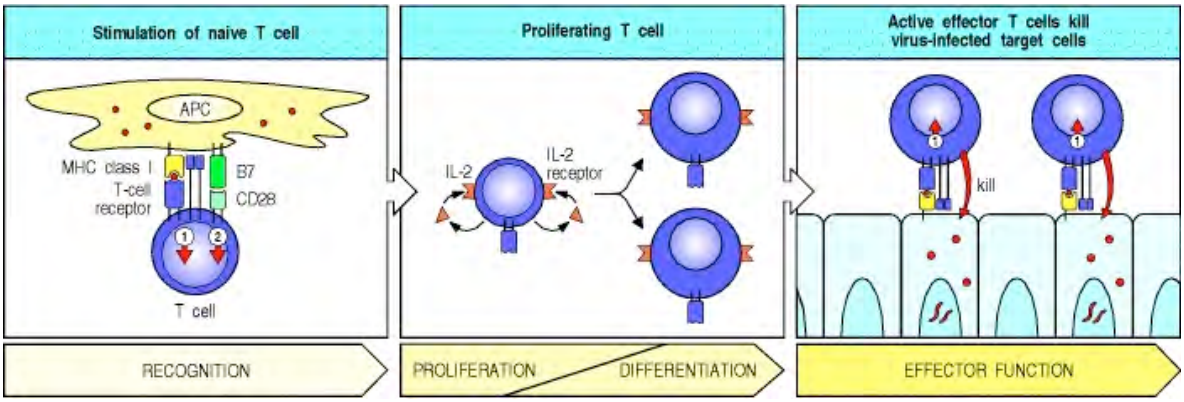


Figure 1. T cell differentiation. (Left) a T cell recognizes antigen presented by an APC and receives the required receptor stimulation (arrows 1: TCR bound to MHC class I with antigen; and 2: CD28 co-stimulation) to initiate (Center) IL-2 driven proliferation and differentiation. (Right) Stimulated CD8 T cells are then capable of recognizing antigen on infected cells and through their effector functions kill the target cells. (Janeway CA Jr, T.P., Walport M, et al., *Immunobiology: The Immune System in Health and Disease*. 5th ed. 2001, New York: Garland Science.)

Engagement of the TCR and costimulatory receptors triggers activation of a complex downstream signaling cascade. The TCR signaling cascade triggers the proliferation and differentiation of the T cell, enabling it to gain effector functions. These effector functions depend on the type of co-receptor present on the T cell. $CD4^+$ T cells become one of several types of helper T cells, which respond to exogenous pathogen antigen and upon activation produce cytokines and chemokines that both attract other immune cells to the site of infection as

well as activate those cells [5]. $CD8^+$ T cells develop into cytotoxic T cells, which are critical to identifying host cells infected with intracellular pathogen and secreting cytolytic chemicals that cause apoptosis of infected cells they are bound to [5]. T cell development of effector functions is critical to the adaptive immune response, as helper T cells enhance the activation of APCs, especially B cells, and other immune system cells through the secretion of cytokines, such as IL-2, IL-4, and IFN-γ, which among other purposes, promote cell proliferation and differentiation (Figure Intro-1, Intro-2, and Intro-14) [6-9].

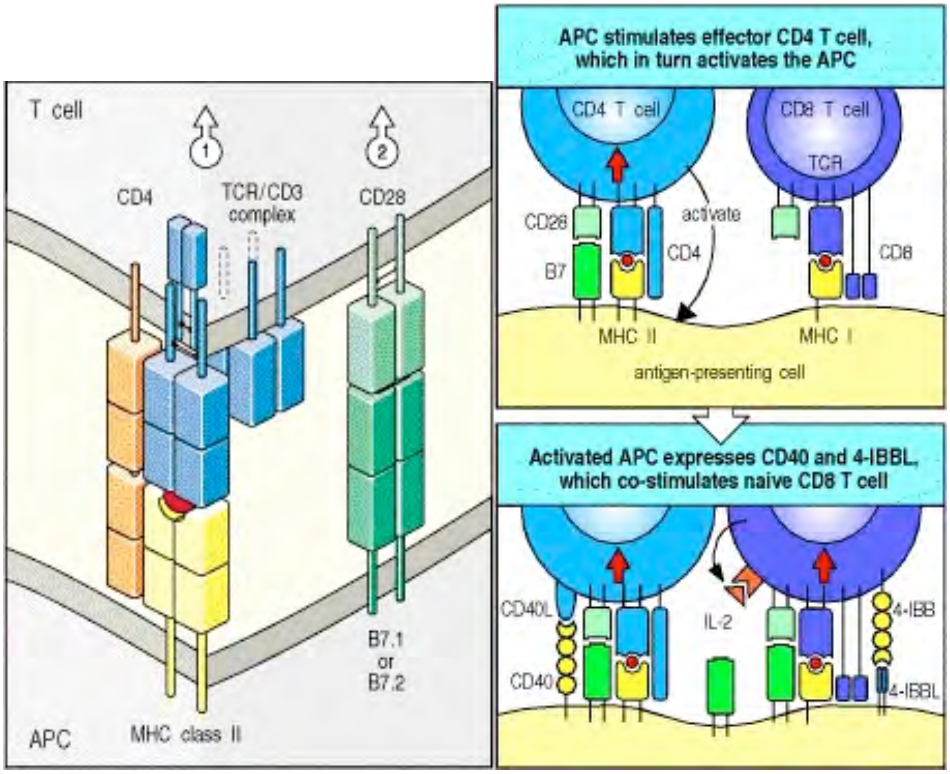


Figure 2. T cell and antigen presenting cell, cell surface receptors and ligands. (Left) Diagram of the T cell receptor (TCR) on $CD4^+$ T cells binding to antigen on APC presented by MHC class II with co-stimulation by CD28 and B7. (Right) $CD4^+$ helper T cells upon TCR stimulation facilitate activation of nearby APCs, which then can provide the co-stimulatory signals required for CD8 cytotoxic T cell activation. (Janeway CA Jr, T.P., Walport M, et al., *Immunobiology: The Immune System in Health and Disease*. 5th ed. 2001, New York: Garland Science.)

Initiation of signal transduction via the T cell receptor

Activation of the T cell is dependent on binding of a specific TCR to its target peptide antigen, presented by the MHC complex on the surface of an APC. In addition to the TCR, the T cell requires binding of a co-receptor (e.g. CD4 or CD8) and co-stimulation via CD28 to induce cellular activation. Binding of antigen by the TCR with cooperative binding by co-receptors initiates a cascade of events that includes the enrichment of receptors at the T cell-APC junction, called the immunological synapse (IS) [10]. The IS serves as a "command post" where central and peripheral signaling occurs in a bullseye-pattern known as the supramolecular activation cluster (SMAC). At the SMAC, intracellular signaling molecules can more effectively be recruited to the activated TCR and transduce downstream signals with greater efficiency (Figure Intro-3) [11]. Once the binding of antigen occurs, the intracellular domains of the TCR initiate the recruitment of Fyn and Lck, Src kinases, which cluster and activate the immunoreceptor tyrosine-based activation motifs (ITAMs) on the TCR [12-14]. Phosphorylation of ITAMs induces recruitment of ZAP-70, a kinase that is phosphorylated and activated by Lck. ZAP-70 then phosphorylates additional downstream adaptor molecules including SLP-76 and LAT. LAT and SLP-76 act in conjunction to activate phospholipase C-gamma (PLC- γ). Cleavage of PIP_2 by PLC-γ yields diacylglycerol (DAG) and IP_3, creating a rise in intracellular calcium levels that then causes calcium channels in the cell membrane to open creating an extracellular Ca^{+2} influx [15]. This spike in intracellular calcium activates calcineurin, which dephosphorylates the transcription factor nuclear factor of activated T cells (NFAT) [16]. NFAT is retained in the cytoplasm by the masking of its nuclear localization signal (NLS) by phosphorylation; however, upon dephosphorylation this domain is uncovered and its nuclear export signal (NES) is then masked, allowing NFAT to translocate to the nucleus [8].

In addition to functioning in the NFAT signaling cascade, DAG activation leads to the downstream regulation of the Ras/MAPK (mitogen activated protein kinase) pathway, which activates Fos [17]. Together with the downstream activation via CD28 co-stimulation and the JNK pathway activation of Jun, heterodimers of Fos and Jun bind to form AP-1 [18]. AP-1, a highly variable heterodimeric transcription factor, is then able to travel to the nucleus [19]. NFAT and AP-1 can act individually or cooperatively on their respective gene promoter regions. NFAT and AP-1 are required transcription factors for many cell types besides T cells for their regulation of cellular activation, proliferation, and differentiation. They are especially important in T cells for their role in the expression of cytokines, such as IL-2 [8, 20]. Finally, DAG also aids in protein kinase C-theta (PKC-θ) recruitment to the plasma membrane and the TCR while PDK-1 acts to phosphorylate and activate PKC-θ [21].

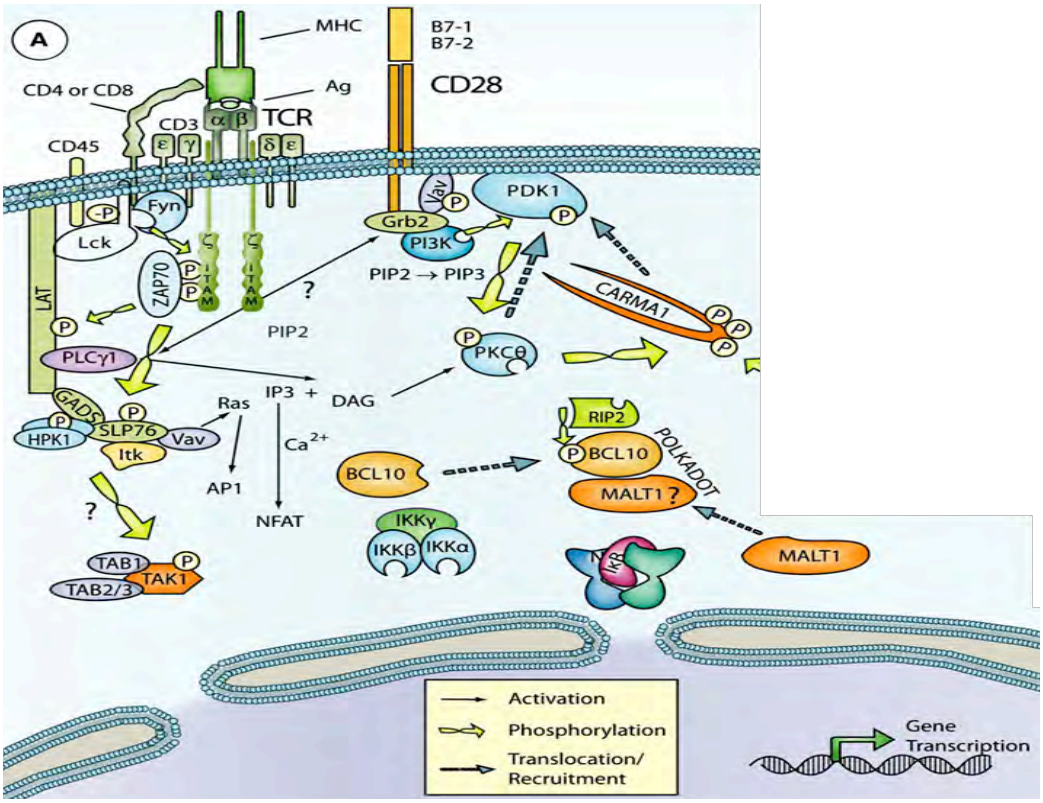
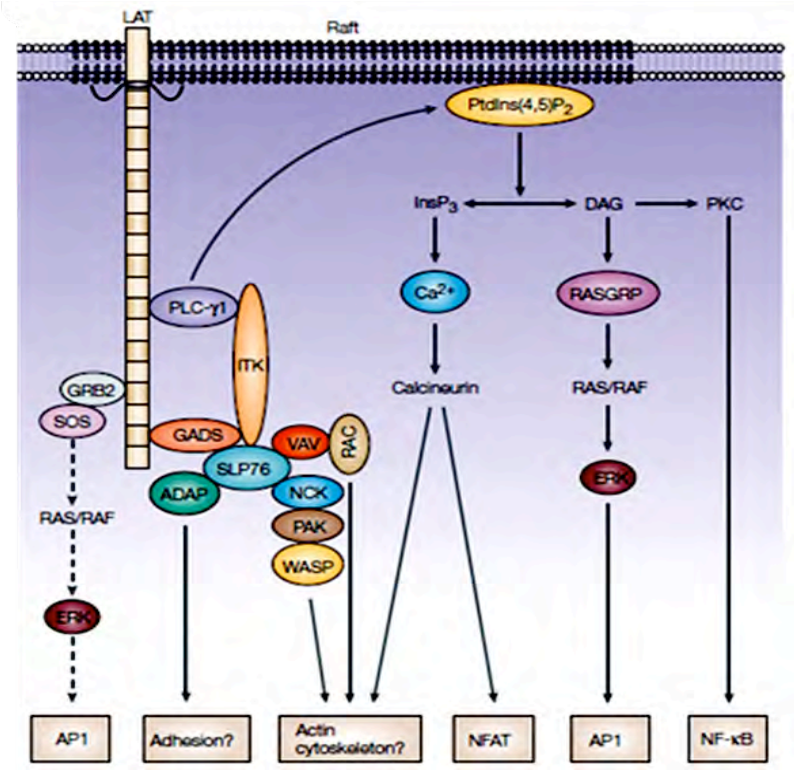


Figure 3. Initial signaling stages of TCR stimulation in a naïve T cell. A cascade of tyrosine phosphorylation proteins is the starting point of several signaling pathways that lead to the activation of key transcription factors (e.g. NF-κB). Activation of PKC-θ in T cells facilitates phosphorylation and activation of CARMA1, which can then bind to the ubiquitously associated proteins Bcl10 and MALT1, forming the CBM complex in the context of a POLKADOT (Schaefer 2004). (Schulze-Luehrmann, J. and S. Ghosh, *Antigen-receptor signaling to nuclear factor kappa B.* Immunity, 2006. **25**(5): p. 701-15.)

Figure 4. Transcription factors downstream of the TCR. A signalsome assembled along a cellular membrane lipid raft helps to facilitate binding of and activation of signal cascade proteins that lead to downstream activation of multiple cellular processes including the transcription factors NFAT, AP-1, and NF-κB. (Horejsi, V., W. Zhang, and B. Schraven, *Transmembrane adaptor proteins: organizers of immunoreceptor signalling.* Nat Rev Immunol, 2004. **4**(8): p. 603-16.)



The third critical transcription factor activated as a result of TCR stimulation is nuclear factor κB (NF-κB), which is ubiquitously expressed in all cells and can be activated downstream of PKC in every cell type [9, 22]. Thus, there are three centrally important transcription factors that are activated downstream of the TCR through signaling cascades: NFAT, AP-1, and NF-κB (Figure Intro-4). These three transcription factors act in concert to induce and regulate transcription of genes that control cell activation, proliferation, and differentiation in not only T cells but a multitude of cell types.

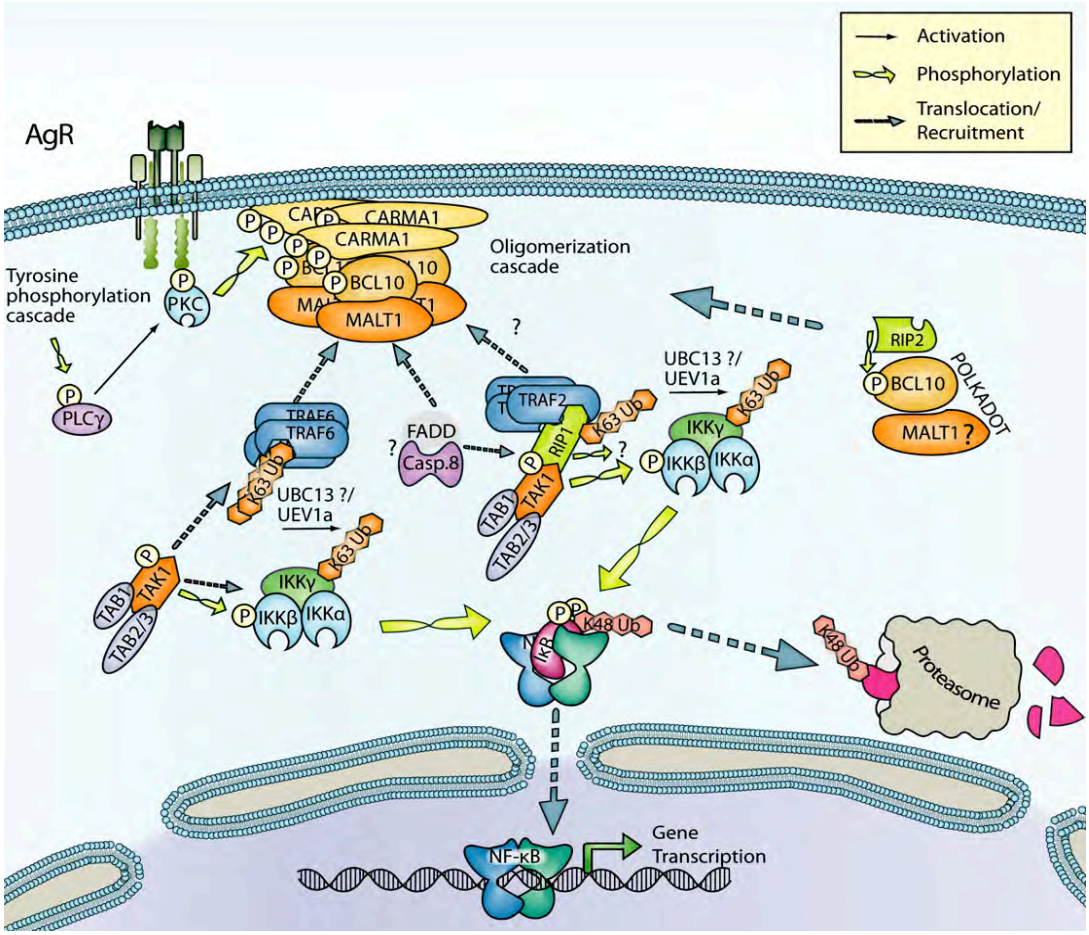


Figure 5. Intermediate signaling downstream of the TCR. The NF-κB signaling cascade includes multiple types of protein modification including phosphorylation, K48-linked and K63-linked ubiquitination. The CBM complex in response to phosphorylation translocates to the cell membrane where further downstream signaling occurs through recruitment of additional signaling molecules, such as TRAF6. Subsequent phosphorylation of the IKK complex by TAK1 and ubiquitination of IKK-γ allows it to K48 ubiquitinate the IκB complex, releasing NF-κB. NF-κB dimers then translocate to the nucleus while IκB is degraded by the proteasome. (Horejsi, V., W. Zhang, and B. Schraven, *Transmembrane adaptor proteins: organizers of immunoreceptor signalling.* Nat Rev Immunol, 2004. **4**(8): p. 603-16.)

NF-κB pathways of activation

The transcription factor NF-κB consists of two subunits that form either homo- or hetero-dimers [6, 7]. There are five subunits in the NF-κB family of proteins, which includes p65 (RelA), RelB, cRel, p52 (p100), and p50 (p105). These five proteins dimerize through their Rel homology domain (RHD), with each subunit containing a DNA binding domain that allows the NF-κB dimer to bind to specific DNA motifs. In addition to the RHD and DNA binding domain, p65, RelB, and cRel also contain a transcription activating domain. NF-κB activation proceeds via two distinct classes of signaling cascades, known as the canonical and non-canonical pathways (Figure Intro-7 and Intro-14) [23, 24].

The NF-κB non-canonical pathway occurs through engagement of specific members of the TNF receptor family, such as CD40, and involves the activation of NF-κB inducing kinase (NIK) [23]. NIK then activates the IKK complex subunit alpha (IKK-α) [24]. This activation of the IKK complex leads to subsequent phosphorylation of the NF-κB subunit by IKK-α, which then causes ubiquitination and proteasomal processing of the protein from p100 to p52. This NF-κB subunit can then dimerize with a second subunit, RelB, to form NF-κB and translocate to the nucleus. The focus of this thesis, however, is the NF-κB signaling pathway downstream of the TCR, which proceeds via the canonical pathway.

T cell receptor activation of the NF-κB cascade

The NF-κB canonical pathway in T cells is initiated by the key mediator PKC-θ [25], which activates downstream NF-κB signaling cascade intermediates by phosphorylating CARMA1 to its functionally active form (Figure Intro-5). Conformational change of CARMA1 incurred through phosphorylation of its linker domain via PKC-θ [26] destabilizes an intramolecular association with its caspase recruitment domain (CARD) [27-29]. After activation CARMA1 adopts an "open" conformation, enabling binding to Bcl10 through their respective CARD domains and binding to MALT1 through its coiled-coil domain directly downstream of the CARD domain [26, 30, 31]. This CARMA1-Bcl10-MALT1 (CBM) complex (Figure Intro-8) [26, 32] is then a staging point for further protein-protein interactions (Figure Intro-5). In response to TCR activation, the CBM complex forms punctate structures within the cytoplasm, called POLKADOTS (punctate and oligomeric-like killing or activating domains transducing signals) [33]. POLKADOTS are enriched sites of signaling intermediates primarily associated with the CBM complex as well as those proteins linked with CBM complex modifications, such as phosphorylation of Bcl10 by RIP2 (Figure Intro-5) [34, 35]. Overtime, the POLKADOTS migrate from the cytoplasm to a region beneath the SMAC, where they become coalesced (Figure Intro-5).

As a whole, the CBM complex represents a major point of dynamic signaling, involving both protein alteration and spatial redistribution [36]. MALT1, in addition to containing a functional caspase-like protease domain [37], is important in recruiting and binding to the TNF-associated factor 6 (TRAF6), a ubiquitin ligase that functions in K63-linked ubiquitination of Bcl10 and MALT1, which promotes IκB kinase (IKK) complex recruitment (Figure Intro-5) [38, 39]. The IKK complex consists of three subunits: alpha, beta, and gamma (NEMO) [40]. The IKK complex is activated through K63-linked ubiquitination of its NEMO subunit, which

enables recruitment of TAK1 a kinase that phosphorylates the catalytic subunit IKKβ (Figure Intro-5) [41]. IKK-γ ubiquitination and phosphorylation of IKK-β by TAK1 [41] activates the IKK complex, allowing it to phosphorylate inhibitor of κB (IκB) [42]. Prior to activation, NF-κB dimers remain localized in the cytoplasm by the masking of its NLS by IκB. IκB, consisting of a family of 6 different proteins, undergoes phosphorylation by IKK, which targets IκB to degradation by the proteasome, subsequently freeing the NF-κB dimers to translocate to the nucleus (Figure Intro-5).

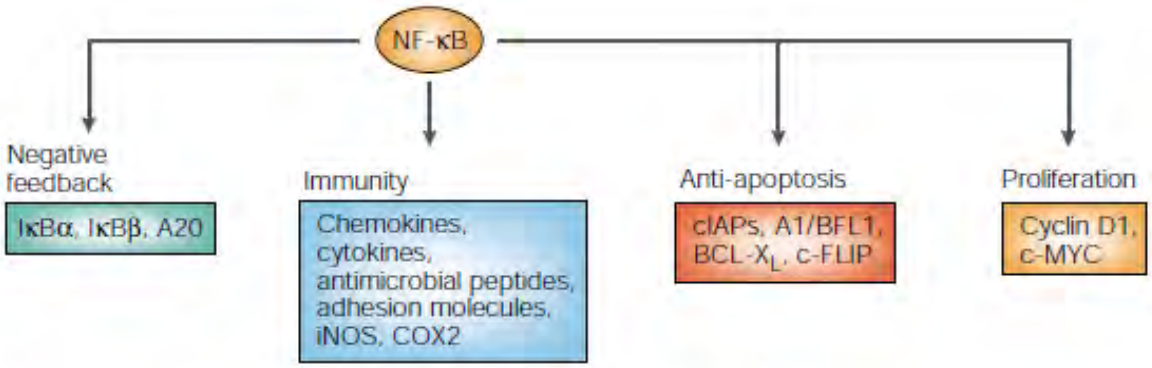


Figure 6. Transcription factor properties of NF-κB. The transcription factor NF-κB controls the expression of proteins, which function in multiple cellular processes critical for cell regulation and development. (Karin, M., et al., *NF-kappaB in cancer: from innocent bystander to major culprit.* Nat Rev Cancer, 2002. **2**(4): p. 301-10.)

Within the nucleus, NF-κB has a dual function, first, as a transcription factor binding to its target DNA consensus sequences for the purpose of enhancing the promotion and transcription of key genes required for cell proliferation and differentiation (Figure Intro-6) [43]. And second, by driving the transcription of genes to initiate the negative feedback loop of NF-κB to cease the signals leading to cellular activation (Figure Intro-6) [44]. This includes the transport of NF-κB back to the cytoplasm by IκB (a gene induced by NF-κB) through its NES [45].

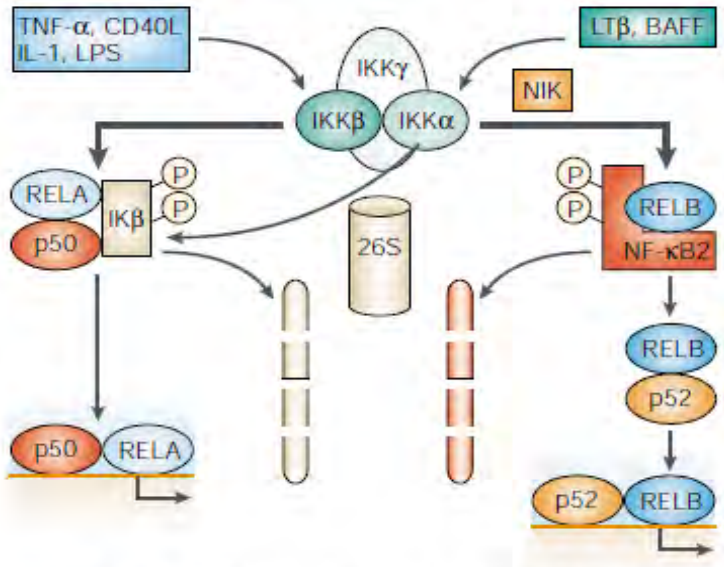


Figure 7. Pathways of NF-κB activation. The canonical (left) and non-canonical (right) pathways of NF-κB activation. (Karin, M., et al., *NF-kappaB in cancer: from innocent bystander to major culprit.* Nat Rev Cancer, 2002. **2**(4): p. 301-10.)

CARMA1 and MALT1 are key intermediaries of NF-κB signaling

As previously mentioned, CARMA1, Bcl10, and MALT1 form the CBM (CARMA1, Bcl10, MALT1) complex (Figure Intro-8). CARMA1 (Figure Intro-8; also known as CARD11, CARD-MAGUK1, Bimp3), an essential NF-κB signaling mediator, is a MAGUK protein family member. The MAGUK family of proteins is characterized by a set of three domains: the PDZ domain, a Src homology 3 (SH3) domain important in binding to lipid rafts, and a guanylate kinase (GUK) domain. CARMA1 has a caspase recruitment domain (CARD), which is a domain found in several types of proteins of which there are four subgroups (as defined by Bouchier-Hayes and Martin) [46]. The CARD domain enables homo and hetero-dimerization of proteins through their CARD domains [47]. CARD proteins are also expressed in a cell-type specific

manner, with CARMA1 being found in hematopoetic cells while other CARD proteins function in the NF-κB signaling cascade of other cells [32], such as CARMA3 (CARD10) in non-immune cells [48, 49].

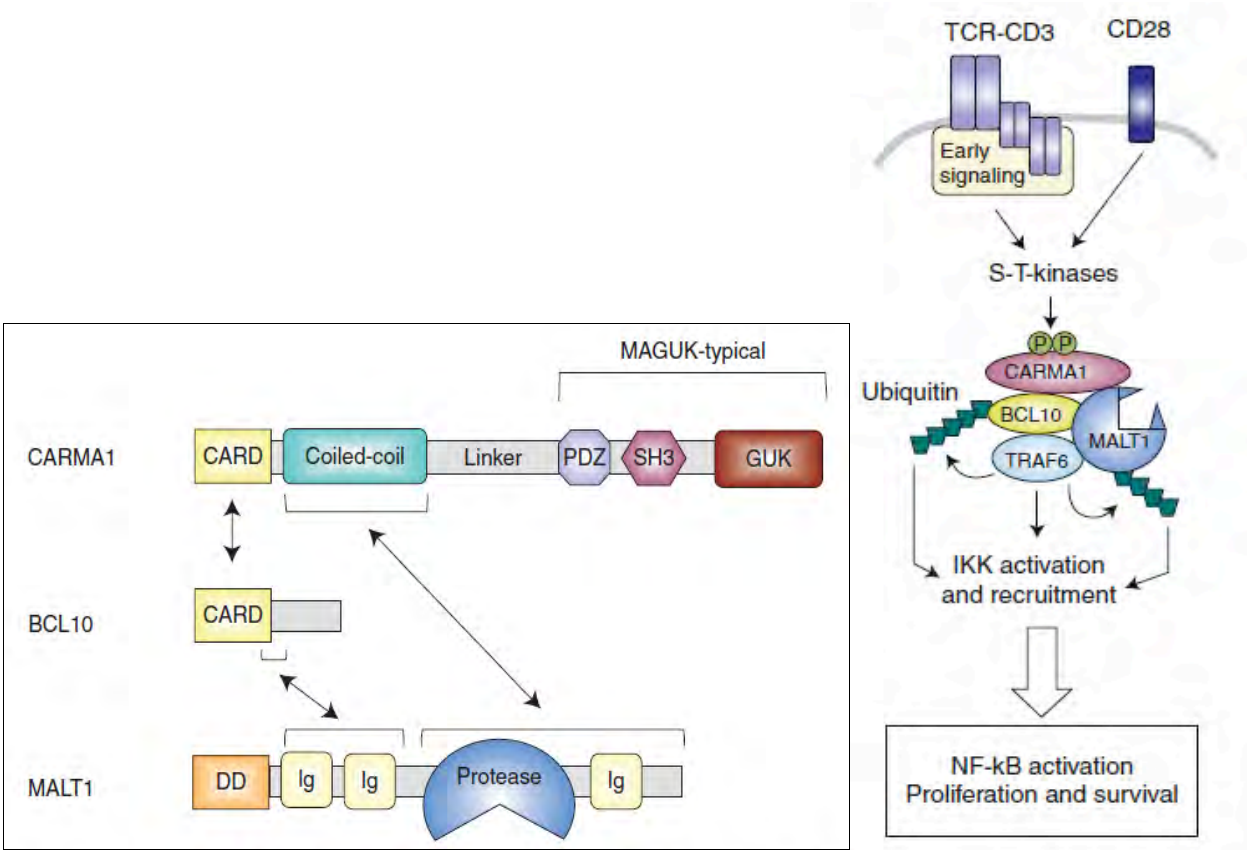


Figure 8. The CBM complex. (Left) The protein domains of the CBM complex members. CARD: Caspase recruitment domain; PDZ: PSD95, DLG, ZO1 homology; SH3: Src homology 3; GUK: guanylate kinase; DD, death domain; Ig: immunoglobulin-like. (Right) The CBM complex in the context of the NF-κB signal cascade with transient modification by phosphorylation and ubiquitination shown. (Thome, M., et al., *Antigen receptor signaling to NF-kappaB via CARMA1, BCL10, and MALT1.* Cold Spring Harb Perspect Biol, 2010. **2**(9): p. a003004.)

MALT1 (Figure Intro-8) a paracaspase found diffusely in the cytoplasm was first discovered due to a chromosomal translocation in mucosa-associated lymphoid tissue (MALT) lymphoma, which was correlated with NF-κB dysregulation [50]. MALT1, in addition to being constitutively associated with Bcl10 through its immunoglobulin-like domains in unstimulated

cells, is also recruited to the CARMA1 coiled-coil domain with Bcl10 via its C-terminal protease and third Ig domain [36, 51]. As previously mentioned, MALT1 also has a functional caspase-like protease domain [37] important in recruiting and binding to TRAF6, which facilitates K63-linked ubiquitination of Bcl10 and MALT1 [38, 39].

An introduction to Bcl10

Another protein initially characterized in MALT lymphomas was named B cell lymphoma 10 (Bcl10; also known as CIPER, CLAP, CARMEN) [52]. Bcl10 (Figure Intro-8) is essential for the NF-κB signaling cascade in T cells and B cells [53]. Bcl10 is a 233 amino acid protein, which interacts with CARMA1 through its CARD domain upon TCR stimulation, but can be found constitutively bound to MALT1 through a minimal MALT1 binding site located at the C-terminus of its CARD domain and extending into a 13-amino acid stretch (amino acids 107-119) just downstream of the CARD, in the serine threonine-rich C-terminus [30, 54]. A third domain of Bcl10 that has been largely overlooked in previous studies consists of a twelve amino acid peptide (Bcl10 amino acids 1-12) just upstream of the CARD domain, dubbed the N-terminal peptide. Although this peptide shows no sequence homology to other known proteins it is well conserved over multiple species (Figure Intro-9).

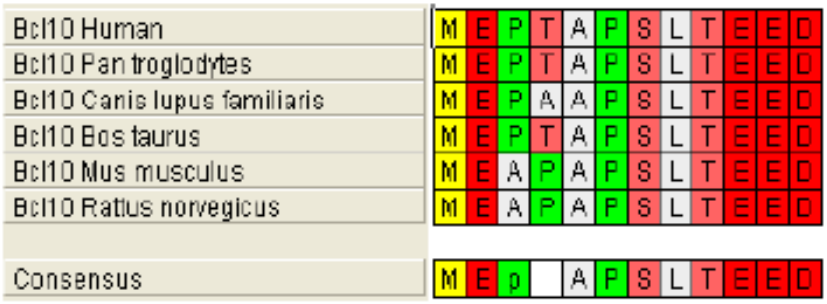


Figure 9. Conservation of the Bcl10 N-terminal peptide. Clustal W alignment of the N-terminal peptide of Bcl10, consisting of the first twelve amino acids of the protein. The consensus sequence over six species indicates this domain is well conserved. (*Thompson, J.D., D.G. Higgins*, and *T.J. Gibson*., *CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice </clustalw/clustalw.html>.* Nucleic Acids Research, 1994.)

Along with many other proteins in the NF-κB signaling cascade, Bcl10 undergoes modification and spatial redistribution (Figure Intro-5) [55]. Bcl10 phosphorylation serves to alter protein function within the NF-κB signaling cascade as well as within other cellular processes (Figure Intro-10), such as phosphorylation on Serine138 and its effect on Bcl10 in actin polymerization and T cell spreading [56]. Within the NF-κB signal cascade Bcl10 phosphorylation has been found to both positively regulate activation via phosphorylation by RIP2 [57], and negatively regulate activation via phosphorylation by IKKβ or CaMKII [58].

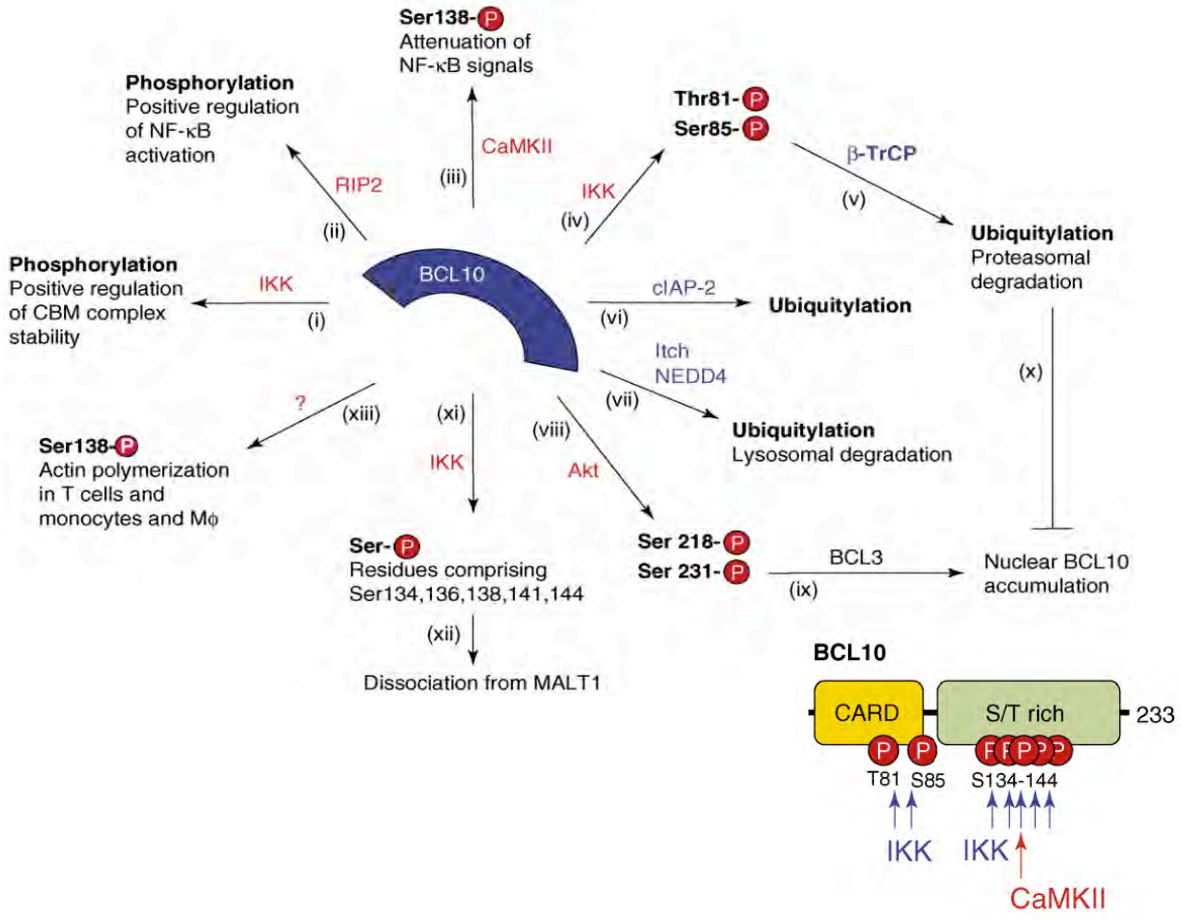


Figure 10. Phosphorylation sites on Bcl10. A review of the possible sites and types of phosphorylation and ubiquitination Bcl10 undergoes via various signaling mediators and the effect of these modifications on Bcl10 function. (Thome, M. and R. Weil, *Post-translational modifications regulate distinct functions of CARMA1 and BCL10.* Trends Immunol, 2007. **28**(6): p. 281-8.)

Bcl10 is also known to undergo both K48- and K63-linked polyubiquitination. K63-linked polyubiquitination alters Bcl10 function, such as through the recruitment of the IKK subunit NEMO [39, 59]. Bcl10 degradation follows a specific mechanistic timeline that has been characterized as both proteasomal and lysosomal depending on its immediate function in the cell at a given time point [60-62]. Phosphorylation on Bcl10 amino acids Thr81 and Ser85 (Figure Intro-10) leads to K48-linked ubiquitination by β–transducin repeat-containing protein (β-TrCP) targeting Bcl10 for degradation by the proteasome [60]. Another study by Scharschmidt *et al* [62] has observed Bcl10 degradation via the lysosomal pathway (Figure Intro-11) upon ubiquitination by the NEDD4 and Itch E3 ubiquitin ligases. A third mechanism of Bcl10 degradation recently studied in our lab by Paul *et al* [61] outlines a TCR-dependent Bcl10 degradation pathway via selective autophagy. Autophagy, a process often seen in nutrient-deprived cells, is the route by which the cell reclaims certain cellular elements for digestion and reuse. Selective autophagy of Bcl10 downstream of TCR stimulation as a means of abating downstream NF-κB activation is a somewhat novel approach to terminating a signal cascade, and has been observed in only few signaling pathways to date [61, 63, 64]. This process of selective autophagy involves the binding of Bcl10 by p62, an autophagy adaptor protein that enables the formation of an autophagosome around Bcl10 selectively excluding MALT1 [61]. Summarily, activation of the NF-κB pathway initiates a negative feedback loop that includes the degradation of Bcl10, which aids in the termination of further activation of the signal cascade downstream of the CBM complex.

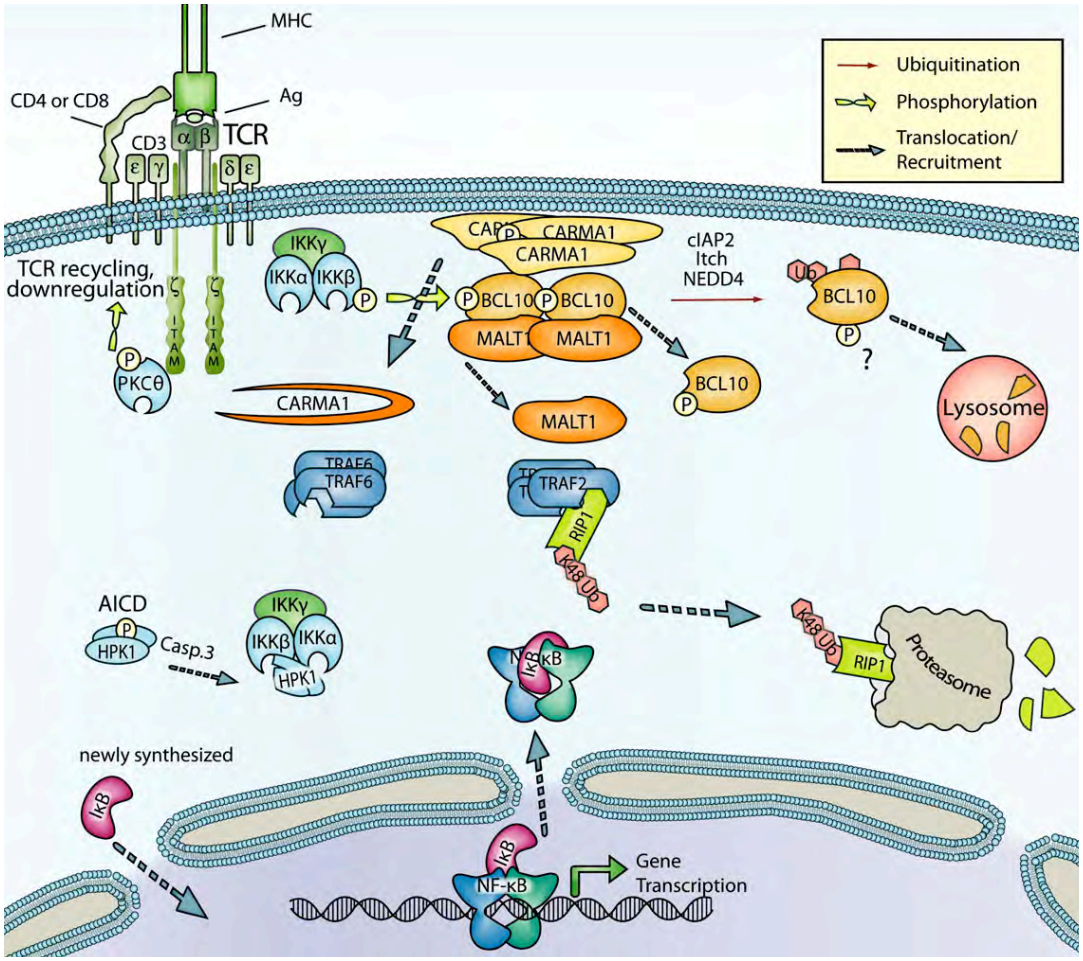


Figure 11. Final stages of NF-κB activation. Initiation of the negative feedback loop of the pathway. The NES of newly synthesized IκB allows the protein to bind nuclear NF-κB and export it back to the cytoplasm. Other pathway mediators undergo disassociation and degradation, such as the members of the CBM complex. Bcl10 undergoes several stages of alteration including proteolytic cleavage via MALT1, phosphorylation by IKK-β, and ubiquitination leading ultimately to its degradation. (Horejsi, V., W. Zhang, and B. Schraven, *Transmembrane adaptor proteins: organizers of immunoreceptor signalling.* Nat Rev Immunol, 2004. **4**(8): p. 603-16.)

In addition to ubiquitination by MALT1 recruited TRAF6 [39], it is believed that MALT1 also proteolytically cleaves Bcl10 (Figure Intro-11) [37], which creates a Bcl10 isoform that functions in the process of T cell adhesion and IS formation [65]. Due to the dynamic nature of Bcl10 modifications, the protein also spatially redistributes within the cell upon activation of the NF-κB pathway [55]. Previous studies have detailed Bcl10 as trafficking both within the cytoplasm [33, 34, 60] and across the nuclear membrane [66-68]. Studies in this lab [33, 34]

have described sites of enrichment for Bcl10 and associated signaling molecules termed POLKADOTS (Figure Intro-12). Bcl10 and members of the CBM complex coalesce in these POLKADOTS, which act as both stable points of protein enrichment and dynamic protein exchange. These POLKADOTS structures migrate over time [34] to the IS, another site rich in signaling intermediates including CARMA1-rich lipid rafts (Figure Intro-12) [69]. Upon TCR stimulation, Bcl10 enrichment at POLKADOTS reaches a peak after approximately 90 minutes. Thereafter, Bcl10 undergoes degradation as discussed above, until POLKADOTS are completely disassociated. The localization of Bcl10 to the cytoplasm may be linked to its ubiquitous association with MALT1 and the presence of two NESs on MALT1, effectively localizing both proteins to the cytoplasm [70]. Bcl10 nuclear enrichment has also been a topic of study as several modes of Bcl10 transport have been proposed, many of which involve Bcl10 phosphorylation [67, 71]. One such example is Bcl10 nuclear translocation by tumor necrosis factor-α (TNF-α)-induced Akt phosphorylation of Bcl10 [68]. The NF-κB family member Bcl3, known to shuttle across the nuclear membrane and act as a transactivator of other NF-κB proteins, has been observed binding phosphorylated Bcl10 and importing it to the nucleus [68]. Bcl10 nuclear enrichment eludes to yet another possible function of Bcl10, that of a transcription factor. A previous study by Chen *et al* [72] indicates that Bcl10 binds to TFIIB through its N-terminal peptide and aids in regulation of gene transcription [73]. Nuclear translocation of Bcl10 has also been proposed as a mode of degradation after TCR stimulation [60]. In addition, the role of Bcl10 in the nucleus has been hypothesized as an important point of possible dysregulation in disease [74, 75] as will be discussed in greater detail later.

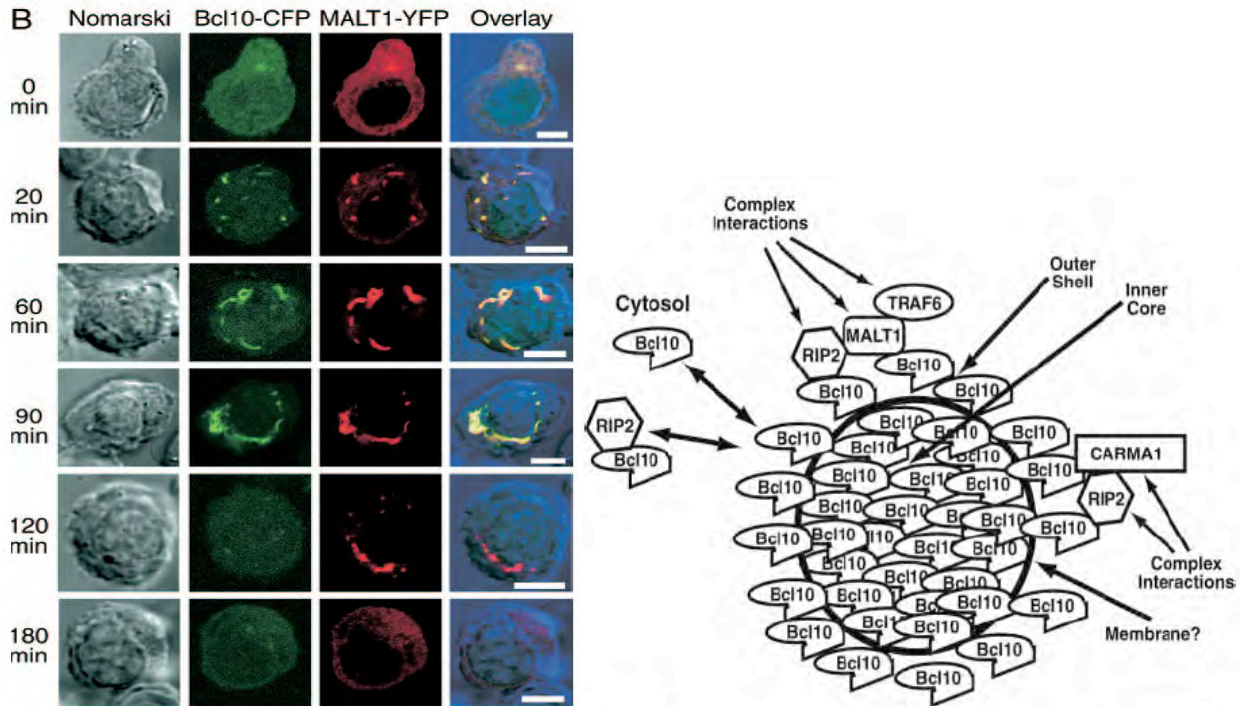


Figure 12. Bcl10 localization to POLKADOTS. (Left) D10 mouse T cells conjugated to CH12 B cells in the presence of their specific antigen conalbumin shows the localization of Bcl10 overtime in conjunction with its CBM partner MALT1. Bcl10 is found diffusely in the cytoplasm in unstimulated cells and then dynamically localizes to several known signaling structures including POLKADOTS (20-60 min) and the SMAC (60-90 min), before becoming degraded and undergoing disassociation from its signaling partners. (Right) A model of POLKADOTS underlining its dynamic interchange of signaling intermediates, such as CARMA1 and RIP2, with a core composed primarily of Bcl10. (Rossman, J.S., et al., *POLKADOTS are foci of functional interactions in T-Cell receptor-mediated signaling to NF-kappaB.* Mol Biol Cell, 2006. **17**(5): p. 2166-76.)

vE10 and its affect on Bcl10 function

Equine herpes virus-2 (EHV-2) protein E10 (vE10) is a homolog of the Bcl10 protein, which is known to disrupt the proapoptotic processes of lymphoid cells in horses [76]. In lymphoid cells infected with this virus, vE10 localizes to the cell membrane and activates downstream NF-κB and JNK signal cascades [77]. vE10 shares a homologous CARD domain with Bcl10, allowing for dimerization of vE10 with Bcl10 similar to that seen with Bcl10 and CARMA proteins [77-79]. However, downstream of their respective CARD domains Bcl10 and vE10 differ quite strongly. The C-terminal domain of vE10 is a glycine-rich region of

approximately 200 amino acids, which is much longer than the Bcl10 serine and threonine-rich C-terminus [77, 79]. This vE10 C-terminal domain contains a geranyl-geranylation domain, which allows vE10 to bind to cell membrane lipid rafts, facilitating the localization of vE10 and any proteins it may bind to, to localize to the cell membrane [77, 80].

Because of the vE10 geranyl-geranylation membrane-tethering domain, vE10-bound Bcl10 is localized to the plasma membrane [80]. Membrane localization causes Bcl10 hyperphosphorylation and Bcl10-mediated activation of downstream NF-κB signaling, dependent on an intact vE10 CARD domain and localization of the protein to the membrane [80]. Much still needs to be learned of the protein vE10, as well as its association with the NF-κB cascade and Bcl10.

Aberrant NF-κB activation is linked to disease

As NF-κB regulates the expression of pro- survival genes and genes regulating cellular growth and differentiation, it is not surprising that dysregulation of this pathway leads to diseases of autoimmunity, immunodeficiency, and cancer (Figure Intro-13). The proteins Bcl10 and MALT1 were both first characterized by translocation mutations found in MALT lymphomas [50, 52]. In the case of Bcl10 for example, a common translocation is t(1;14)(p22;q32), which places the Bcl10 gene under the control of the immunoglobulin heavy chain gene enhancer, causes strong Bcl10 up-regulation [52]. Over expression and unregulated activation of several known NF-κB signaling intermediates has been shown to aberrantly induce the NF-κB signaling cascade [81, 82]. Such dysregulation of the cascade causes NF-κB to constantly positively regulate anti-apoptotic genes such as Bcl-2 [83, 84] and Bcl-x [85]. In addition to unbalanced

cellular growth [43, 86], the role of NF-κB in a cell's ability to gain effector functions means that it is also key to the inflammation response [87], and that over action of this response can lead to autoimmune disorders [88]. As such, NF-κB over activation is associated with a number of cancers and autoimmune disorders ranging from hepatocellular carcinoma [89, 90] to colon cancer (Figure Intro-13) [91] and arthritis [92, 93] to asthma [94-96].

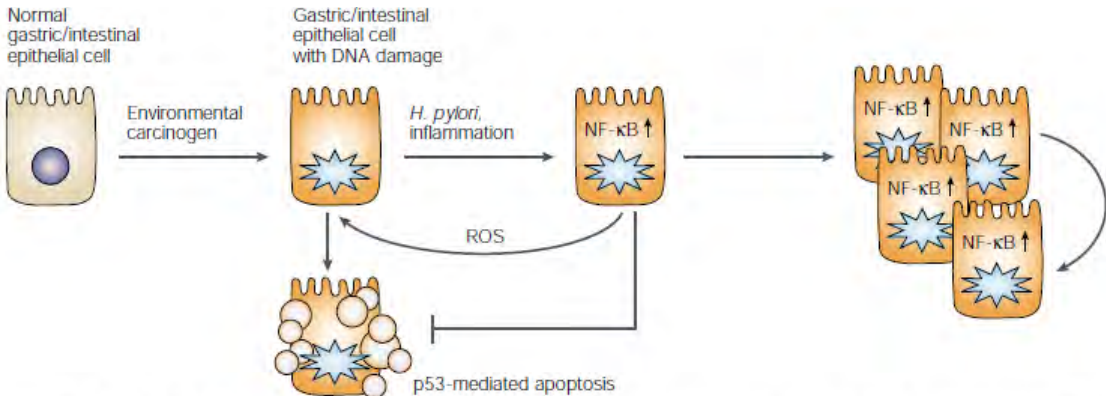


Figure 13. NF-κB dysregulation in disease. One example of NF-κB dysregulation contributing to unchecked cellular proliferation in gastric epithelial cells. (Jani, T.S., et al., *Inhibition of NF-kappaB signaling by quinacrine is cytotoxic to human colon carcinoma cell lines and is synergistic in combination with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or oxaliplatin.* J Biol Chem. **285**(25): p. 19162-72).

Just as important as NF-κB over activation is a lack of NF-κB activation. As previous work in our lab has shown [97], downstream of the TCR, NF-κB activation occurs in a digital (switch-like) manner, with a lack of proper signal strength resulting in an absence of pathway activation. In cells of the immune system, a lack of NF-κB activation can be debilitating to the adaptive immune response as cells would not be able to efficiently grow and gain effector functions in the event of an infection (Figure Intro-14) [7, 98, 99]. It has been shown primarily through knockout mouse studies [100] of various NF-κB signaling intermediates and subunits

that loss of even one protein, such as Bcl10 [53], MALT1 [101], or PKCθ [102], can completely disrupt pathway activation of NF-κB and result in a loss of cellular competency.

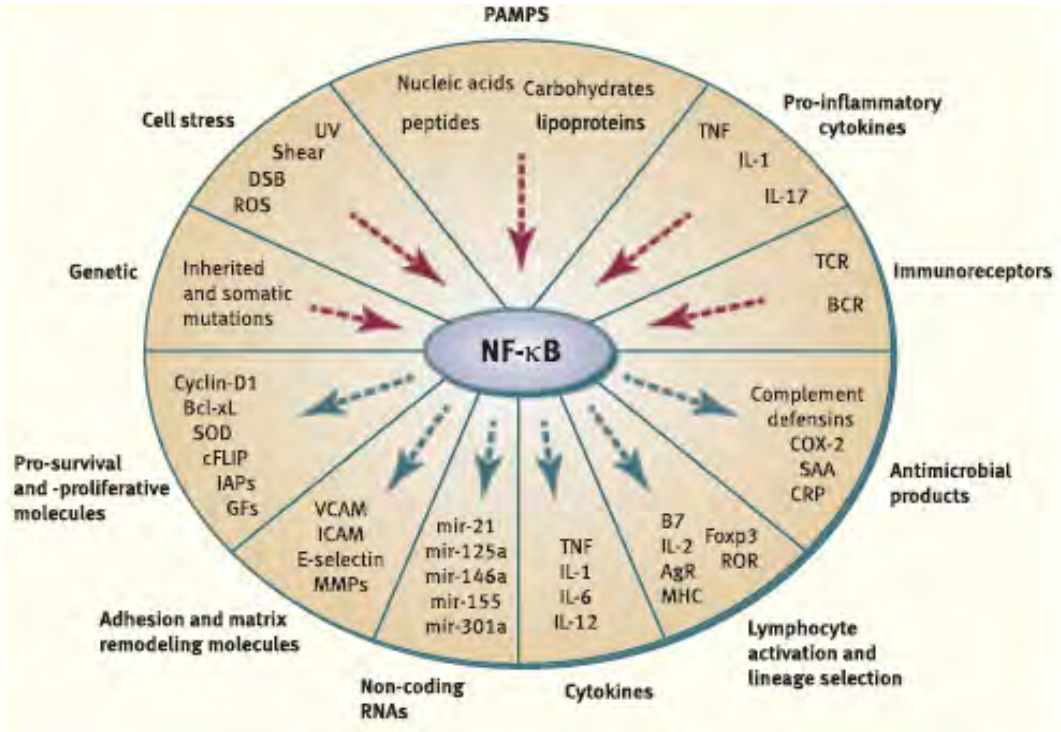


Figure 14. The centrality of NF-κB to cellular functions. A representative sample of pathways leading to NF-κB activation including those of natural cell receptor-based induction as well as cell stresses and mutations (red arrows). Proteins under transcriptional control by NF-κB (blue arrows) are numerous and effect pathways related to cellular survival, cytokine production, and synthesis of antimicrobial products. (Ghosh, S. and M.S. Hayden, *Celebrating 25 years of NF-κB research.)*

Bcl10 has been noted as exhibiting mutations in several diseases [82, 103, 104]. However, whether these variants are mutations that cause aberrant protein function or mere polymorphisms [105-108] has yet to be fully elucidated. One example of Bcl10 protein sequence variability is at the site of alanine five, which in some organisms is replaced by serine, but in humans is believed to contribute to cancer, such as testicular germ cell tumors [104]. Whether the site A5S mutation is linked to an obvious cause of disease is not easily answered as

it is located in the N-terminal peptide directly upstream of the CARD domain, in a region of yet unknown function.

Thesis topics and rationale

There has been much work to define mechanisms of signal transmission in the TCR-stimulated NF-κB signaling cascade. However, much is still unknown regarding some pathway mechanisms concerning protein-protein interactions, protein activation, and protein degradation. Bcl10 is a required protein for NF-κB activation downstream of the TCR as well as other antigen receptors. The full function of Bcl10 within the NF-κB pathway and other pathways however, has not been entirely uncovered. With no crystal structure yet available for Bcl10, it is difficult to understand the conformational changes via phosphorylation and ubiquitination that would alter this adaptor protein's function over the course of cellular stimulation and NF-κB pathway activation. As previously discussed, the N-terminal peptide of Bcl10 is a short twelve amino acid peptide at the Bcl10 N-terminus, directly upstream of the CARD domain. Although some discussion has been given regarding possible phosphorylation [60] and mutation [104] in this region, it is still unknown what function this novel peptide serves in relation to both Bcl10 stability and expression, as well as interaction with partner proteins, such as those of the CBM complex, for downstream NF-κB activation.

Through this study, I sought to fully characterize the function of the Bcl10 N-terminal peptide, both with respect to Bcl10 function and its influence of NF-κB activation. As this novel sequence is well conserved over multiple species, it seemed logical to target the most conserved amino acids for characterization. Through site-directed mutagenesis and deletion I created Bcl10 N-terminal peptide mutants that were either absent of a targeted amino acid sequence or had a

substituted sequence of amino acids. In addition to targeting single and groups of amino acids, I also completely abolished the N-terminal peptide sequence through deletion of amino acids 2-12 or mutation of the peptide to all alanines. Through this methodology I believe there has been established a function for this peptide. As this peptide is directly upstream of the Bcl10 CARD domain, which is pivotal in binding to many other CARD proteins and also MALT1, it was important to characterize Bcl10 behavior with regards to binding to these proteins and the ability of Bcl10 to efficiently traffic within the cell. In addition to observing the N-terminal peptide's properties on Bcl10 behavior, I was interested in observing its effect on downstream NF-κB activation. I believe study of this novel peptide can better define the function of Bcl10 within the TCR-dependent NF-κB signaling cascade. Through this research, I have concluded that the Bcl10 N-terminal peptide plays a critical role in Bcl10 activity, both via influencing binding to partner proteins and regulating Bcl10 stability. These properties may make this novel peptide a viable target for therapeutics related to diseases exhibiting over activation of NF-κB.

Aims

Aim 1

Determine the function of the Bcl10 N-terminal peptide with respect to NF-κB activation

The Bcl10 protein is required for NF-κB activation downstream of the TCR in T cells as well as for NF-κB activation triggered by several other receptors in diverse cell types. It is important to have a thorough understanding of Bcl10 biology for the benefit of revealing the role of Bcl10 in disease. One domain that has been under-studied to date is the N-terminal peptide. Previous research has indicated that this domain may have a critical function in downstream activation of NF-κB. *My hypothesis is that the N-terminal peptide of Bcl10 has a previously uncharacterized site of post-translational modification and/or partner protein binding that is critical for NF-κB activation.* I believe that characterization of this peptide will aid in several areas of research concerned with Bcl10 regulation of NF-κB activity and Bcl10 mutation in diseases characterized by NF-κB dysregulation.

Aim 2

Determine the function of the Bcl10 N-terminal peptide with respect to Bcl10 expression, stability, and modification

Previous research has established that Bcl10 functions in many cellular processes, including downstream NF-κB activation and rearrangement of the cellular cytoskeleton [36, 55]. Bcl10 is degraded upon TCR-induced NF-κB activation, which limits the extent of NF-κB

activation. The infrastructure associated with the regulation of Bcl10 degradation is not fully mapped, with several previous studies being in direct conflict. A recent paper from our lab by Paul *et al* has led to the identification of a selective autophagy-lysosomal proteolysis pathway of K63-polyubiquitinated Bcl10 downstream of the TCR (occurring in effector T cells) [61]. This pathway of Bcl10 degradation is p62-dependent and has been shown to regulate NF-κB activation in a digital fashion, similar to that previously observed in another study performed in our lab by Kingeter *et al* [97].

While it is now known that Bcl10 ubiquitination is critical to degradation by a selective autophagy pathway [61], it is still also degraded in part by the proteasome [109, 110]. Bcl10 phosphorylation targeting Bcl10 for proteolysis may be yet another possible source of Bcl10 regulation [111]. Given the many modifications and complex pathways of degradation previously identified for Bcl10, it is important to also establish whether the N-terminal peptide regulates Bcl10 degradation, and whether binding partners interacting with this domain may affect protein stability.

Experimental Design

Bcl10 mutant design and cloning

Previous research on the N-terminal peptide has been relatively cursory, but several recurring observations include a possible site of mutation or polymorphism within the peptide at A5 and L8 [82, 103, 104] and a possible site of phosphorylation at S7 [60]. Taking into consideration the high degree of sequence conservation (Figure Intro-9) within the N-terminal peptide over multiple species, it appeared that these sites were likely important to the function of the protein, and should therefore be characterized both individually and in grouped subsets. Site-directed mutagenesis of individual and groups of amino acids was performed in order to either delete the amino acid(s) of interest or replace the given site with alanine(s). In the case of amino acid sites of possible phosphorylation, these sites were also replaced with a phosphorylation-mimicking amino acid, such as aspartic or glutamic acid (for serine or threonine, respectively). Additionally the entire N-terminal peptide was either deleted (deletion of amino acids 2-12) or replaced with alanines. And lastly, the N-terminal peptide alone was cloned without any additional downstream Bc10 protein sequence. The functionally impaired G78R Bcl10 construct is a previously described mutation of the CARD domain that prevents proper folding, protein-protein association, and NF-κB activation [112]. G78R was thus used as a negative control for most these experiments. This collection of Bcl10 N-terminal peptide mutants was then fused at the C-terminus to GFP (and one wild-type (WT) construct was generated with GFP fused to the N-terminus). These constructs were cloned into several vectors, including the retroviral vectors MSCV and pEhyg [113], and the expression vector pcDNA3 (with or without a 3xHA tag). GFP

was fused to the Bcl10 C-terminus to avoid any possible steric hindrance associated with the large GFP tag on the N-terminal peptide. However, the WT Bcl10 construct with GFP fused to its N-terminus was designed to test the effect of blocking access of potential N-terminal peptide binding partners.

NF-κB luciferase assay

Human HEK-293T cells were subjected to a modified calcium phosphate method of transfection [114] with pBVI-NF-κB-Luc (gift from Gabriel Nunez), a luciferase reporter driven by an NF-κB-dependent promoter. This assay was used to assess the ability of the N-terminal peptide mutants to activate NF-κB relative to WT Bcl10. Overexpression of Bcl10 is known to induce activation of the NF-κB pathway. HEK-293T cells were transfected with a single MSCV Bcl10 construct (112.5 ng of DNA), the NF-κB inducible luciferase promoter, and a beta-galactosidase reporter gene (pEF1-Bos-βgal; gift from Gabriel Nunez) for transfection efficiency normalization. Luciferase activity in cell lysates was quantified using a luminometer. Fold-activation of NF-κB was calculated by performing all mutant samples in triplicate, then averaging the β-Gal concentration for all sample sets and dividing each β-Gal concentration by the average to normalize the sample results; the positive control luciferase concentrations (those that included the luciferase and β-Gal reporters but no Bcl10 plasmid) were then averaged (essentially making these samples the baseline reading equal to “1”) and each luciferase concentration was then divided by this baseline luciferase concentration average (making all of the luciferase readings a normalized value relative to the baseline control); finally, the normalized luciferase concentrations were divided by the normalized β-Gal concentrations

giving the correct relative luciferase units (RLUs) that translate into fold NF-κB activation for each sample set.

D10 T cell transduction and fluorescence microscopy

TCR stimulation promotes formation of punctate cellular structures, called POLKADOTS, which contain CBM complex members [33, 34]. To visually assess the structure of such POLKADOTS formed by WT Bcl10 *vs.* mutant Bcl10 constructs I used D10 T cells (D10 cells), which are a $CD4^+$ T-helper 2 (Th2) clonal line [115]. D10 cells were stably transduced with a pEhyg Bcl10 viral plasmid and positively selected using the hygromycin antibiotic resistance gene included on the plasmid. These Bcl10 mutant-specific D10 cells were then stimulated with their specific antigen (conalbumin), presented by CH12 mouse B cells (CH12 cells). Conjugated T cells were fixed and mounted on slides at the 20 minute time point, when POLKADOTS are at their peak, and then analyzed using fluorescence microscopy to visualize each Bcl10-GFP construct. The POLKADOTS of T cells expressing mutant Bcl10 were objectively compared to POLKADOTS of WT Bcl10 T cells only when the respective T cells were observed as being in conjugation with CH12 cells (to prevent any fluorescent artifacts not associated with TCR stimulation from possibly being quantified). The comparison of POLKADOTS formation between Bcl10 control and mutant cells was based on the relative shape, size, and quantity of structures seen on average in the cells, as well as the average number of cells exhibiting successful POLKADOTS formation when conjugated to CH12 cells, compared to WT Bcl10 T cells.

Co-immunoprecipitation and western blotting

As downstream TCR activation of NF-κB is dependent on formation of the CBM complex, it was necessary to test the association of Bcl10 mutants with the Bcl10 binding partners CARMA1, MALT1, CARMA3, vE10, WT Bcl10. Using HEK-293T cells, I again transiently transfected cells with one pcDNA3 3xHA Bcl10 construct and one Bcl10 binding partner tagged with a 3xFLAG tag (e.g. CARMA1, MALT1). Protein complexes from cell lysates were then co-immunoprecipitated (co-IP) using an anti-FLAG antibody (to directly pull down the given Bcl10 binding partner). Co-IP samples were then run on a denaturing SDS-PAGE gel, transferred to a nitrocellulose membrane via the semi-dry electroblot method, and western blotted. Protein blots were probed for Bcl10 using an anti-HA antibody and underwent secondary blotting for anti-FLAG to normalize protein pull-down. By this method of immunoprecipitation I could determine how well each Bcl10 N-terminal peptide mutant could bind to each partner protein.

Fluorescence microscopy of D10 Bcl10 T cells co-expressing vE10

D10 T cell lines expressing a Bcl10 construct were co-transduced with vE10 and analyzed within 2 days to prevent cell toxicity due to continuous NF-κB activation. These D10 T cells were incubated with CH12 B cells (unprimed or primed with antigen (conalbumin)), then fixed and mounted on slides as previously described. These conjugated cells underwent an additional step of intracellular antibody staining against the 3xFLAG tag of vE10 using an Alexa647 secondary antibody. Using fluorescence microscopy, Bcl10 was visualized via its

GFP tag and vE10 was visualized via its Alexa647 antibody-conjugated 3xFLAG tag. Using an overlay of each fluorescent image, I was able to compare each N-terminal peptide mutant's cellular localization when bound by vE10 compared to WT Bcl10. Previous studies have shown that vE10 localizes to the cell membrane via a geranyl-geranylation motif and also translocates bound Bcl10 to the membrane, via a CARD-CARD interaction [77]. To determine whether vE10 requires the Bcl10 N-terminal peptide to bind and translocate Bcl10 to the cell membrane, I used fluorescence microscopy to image vE10 subcellular localization relative to both WT and N-terminal peptide mutant Bcl10 constructs in both unstimulated and stimulated T cells.

Cycloheximide assay

To establish the function the Bcl10 N-terminal peptide has on Bcl10 protein stability, D10 T cells and HEK-293T cells were cultured with cycloheximide (CHX), an inhibitor of protein translation. Cells were treated with CHX (0-12 hr; 50 μg) and then cell lysates were run on an SDS-PAGE gel. As described above, gels were transferred and immunoblotted. Blots were probed with an anti-Bcl10 antibody to quantify Bcl10 protein in untreated and cycloheximide-treated cells, allowing comparison of Bcl10 N-terminal peptide mutants with WT Bcl10 cells. To normalize cellular protein levels, the housekeeping gene GAPDH was detected by immunoblotting. Blots were also probed for cyclin D3, a cell cycle regulator critical to the G1/S phase transition that is rapidly degraded prior to S phase entry. The cyclin D3 blot was a control to verify the CHX treatment was working as expected, with a weaker cyclin D3 protein band expected for CHX-treated cells (because protein translation is inhibited and thus the existing protein in the cell is undergoing rapid degradation). By comparing cells treated with

cycloheximide to untreated cells I was able to determine the stability of each Bcl10 construct relative to WT Bcl10. This methodology shows the degradation rate associated with existing steady-state Bcl10 protein when translation of new Bcl10 protein is halted.

MG132, E64d, and Bafilomycin assays

To determine the pathway of Bcl10 protein degradation possibly associated with the N-terminal peptide domain, three reagents were used to block each cellular degradation pathway; MG132, an inhibitor of the proteasome, E64d, an inhibitor of lysosomal proteases, and Bafilomycin A1 (Baf), an inhibitor of lysosomal acidification. D10 T cells and HEK-293T cells were treated with MG132 (25 μM; 20 hours), HEK cells were treated with E64d (10 μM; 20 hours), and D10 T cells were treated with Baf (50 nM; 5 hours) and then lysates were run on an SDS-PAGE gel. Gels were transferred as previously described and western blotted using an anti-Bcl10 antibody. Membranes were stripped and re-probed for GAPDH (a protein loading control) and cyclin D3 (which is rapidly degraded by the proteasome) to note accumulation of protein in MG132 treated cells. Comparing results of identical experiments using treatments of either MG132 or Baf allowed me to determine what function the Bcl10 N-terminal peptide has in Bcl10 degradation by the proteasomal and/or lysosomal pathways, when compared to untreated cells.

Bisindolylmaleimide Assay

To establish the function of the Bcl10 N-terminal peptide with respect to Bcl10 hyper-phosphorylation, the broad spectrum PKC inhibitor Bisindolylmaleimide (BIM) was used to treat both D10 T cells stably expressing Bcl10 retroviral constructs and HEK-293T cells transiently transfected with Bcl10 plasmids, as described above. BIM treatment of D10 T cells was at 10 μM BIM for the time stated and HEK-293T cells were treated at 4 μM BIM for the time stated. Whole cell lysates were collected for both cellular assays and run on an SDS-PAGE gel that was then western blotted. Western blots were probed with antibodies for Bcl10 protein and GAPDH as a loading control. In conjunction with the CHX and MG132 assays, the BIM assay is designed to better understand the degradation kinetics of specific Bcl10 isoforms, specifically the hyperphosphorylated isoforms that are more prevalent in Bcl10 constructs that have a mutated N-terminal peptide (e.g. d2-12 Bcl10). As BIM is a broad spectrum PKC inhibitor, this assay was not designed to determine whether a specific PKC phosphorylates Bcl10, but rather if Bcl10 hyper-phosphorylation is an (in)direct product of PKC phosphorylation and whether inhibition of Bcl10 hyper-phosphorylation downstream of PKC can affect Bcl10 cleavage and/or degradation.

Results

Aim 1

Determine the function of the Bcl10 N-terminal peptide with respect to NF-κB activation

The Bcl10 N-terminal peptide aids in downstream NF-κB activation (Figures 1A and 1B)

Research to date on the Bcl10 N-terminal peptide has centered on screening the whole protein for possible sites of mutations. The N-terminal peptide of Bcl10 has been cited as being a possible site of phosphorylation (S7) [60], as well as containing either two sites of mutation or short nucleotide polymorphisms (SNPs) at sites A5 (A5S) [82, 103, 104] and L8 (L8L) [104]. Implications of these sites of N-terminal peptide mutation have been frequent, but they also often coincided with mutations elsewhere in the Bcl10 protein, making it difficult to determine which mutation, if any, contributed to progression of disease [82, 104]. Site-specific mutation of the N-terminal peptide of Bcl10 eliminated the possibility of additional mutations downstream that may have left previous results as to the N-terminal peptide's role in aberrant Bcl10 function inconclusive.

Using previous research and the fact that the N-terminal peptide has a well conserved amino acid sequence, I performed site-directed mutagenesis to create N-terminal peptide mutants. These mutants characterized nearly every individual amino acid singly or in grouped subsets, either through deletion or mutation to alanine, aspartic acid, or glutamic acid. I used a modified calcium-phosphate method of transfection of individual Bcl10 constructs into HEK-293T cells to assay each mutant's ability to activate NF-κB relative to WT Bcl10. Cellular

overexpression of Bcl10 is known to induce activation of the NF-κB pathway. In addition to transfecting a Bcl10 construct, cells also received a plasmid containing an NF-κB inducible luciferase reporter gene, and a beta-galactosidase reporter for transfection efficiency normalization. At approximately 48 hours post-transfection, cellular lysates were analyzed by luminometer for relative luciferase units (RLUs) as a measurement of NF-κB activation.

A spectrum of NF-κB activation by the Bcl10 N-terminal peptide mutants was observed. However, none achieved the same level of NF-κB activation as WT (Figure 1A and 1B). Using the Bcl10 mutant G78R, which has a mutation in the CARD that prevents proper folding, as a negative control for NF-κB activation, I was able to judge the N-terminal peptide mutants as either being unable to activate NF-κB (RLUs similar to that of G78R mutant) or activating NF-κB on a scale comparative to WT Bcl10. Figure 1A shows that three N-terminal peptide mutants (1-12 only, d2-12, and All-Ala), clearly exhibit an inability to activate NF-κB. A fourth mutant (EED>AAA) contains a missense mutation of the last three amino acids directly before the functionally critical CARD domain of Bcl10. This mutant also causes a significant impairment of NF-κB activation that is barely above baseline activation levels. Progressive deletion of the N-terminal peptide starting with the N-terminus of the domain (e.g. mutant d2-4) shows an impairment of NF-κB activation congruent with the length of the deletion. Starting with deletion of three amino acids (mutant d2-4), there is an approximately 30% reduction in NF-κB activation; the d2-9 mutant (eight amino acid deletion) exhibits an approximate 60% reduction in NF-κB activation relative to WT; and finally the full d2-12 mutant, is unable to activate NF-κB. The WT Bcl10 construct that has its GFP tag at the N-terminus of the protein directly upstream of the N-terminal peptide also shows an approximately 60% impairment in NF-κB activation relative to WT Bcl10 with a C-terminus GFP tag. Lastly, of the potential N-terminal peptide

phosphorylation sites identified, I observed that all mutants showed an impairment of NF-κB activation that is apparently unrelated to phosphorylation (Figure 1B and data not shown). This is best illustrated by two double mutants of the prospective phosphorylation sites of S7 and T9, which, when mutated to phosphorylation mimicking amino acids (S7D and T9E), showed no difference in activity *vs.* the phosphorylation-impaired mutants (S7A and T9A).

Collectively, these NF-κB reporter assays indicate the N-terminal peptide of Bcl10 does function in downstream NF-κB activation. The comparison of the d2-12 mutant to the all alanines (All-Ala) mutant confirms that it is not the length of the peptide alone that is critical to Bcl10 functionality but rather elements of individual or subgroups of amino acids. Likewise, the 1-12 only Bcl10 mutant demonstrates that the N-terminal peptide alone cannot affect NF-κB activation. Given the polarized nature of several N-terminal peptide amino acids it is likely they may serve as points of interaction or binding to other cellular molecules or to Bcl10 itself. Together, these data show the Bcl10 N-terminal peptide, a novel domain with no known homologous domains in other proteins, is required for Bcl10-mediated activation of NF-κB.

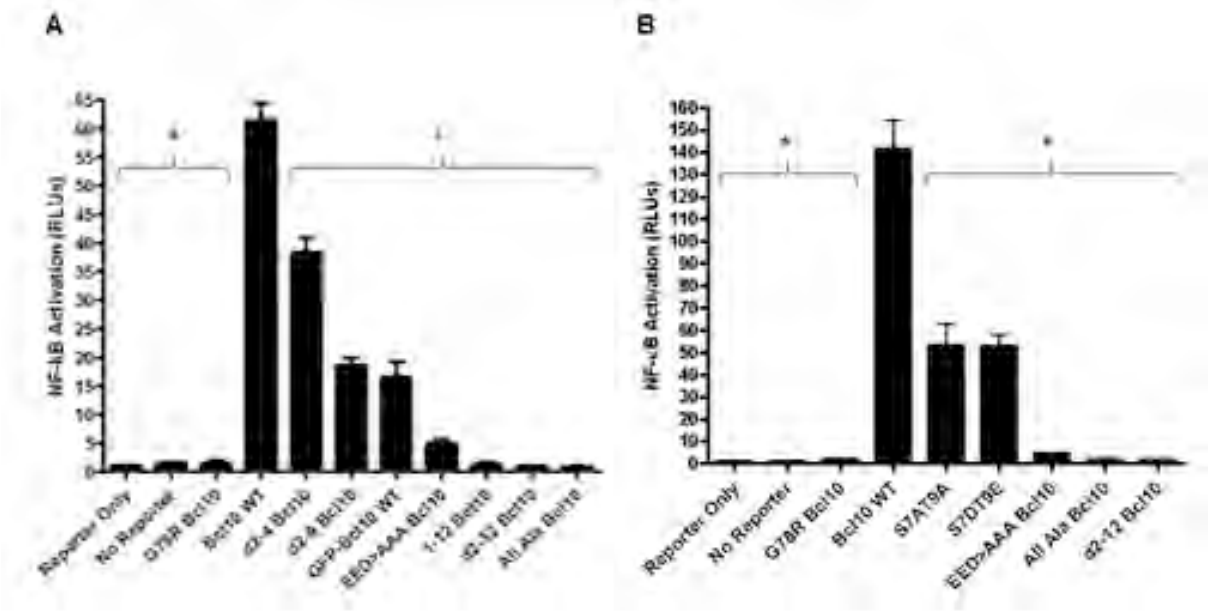


Figure 1: The Bcl10 N-terminal peptide positively regulates Bcl10 NF-κB activation. (A, B) HEK-293T cells were transiently transfected with a single MSCV Bcl10 construct, the NF-κB inducible luciferase promoter, and a beta-galactosidase reporter gene for transfection efficiency normalization. Luciferase activity in cell lysates was quantified using a luminometer. Fold-activation of NF-κB in relative luciferase units (RLUs) was calculated by performing all mutant samples in triplicate, correcting these values by each sample's normalized β-Gal concentration, and then dividing these values by the average value of Reporter Only samples. One-way ANOVA P values (*) represent significance compared to WT Bcl10 ($p < 0.01$).

TCR-stimulated Bcl10 cellular translocation is visibly altered when the N-terminal peptide is mutated (Figures 2A and 2B)

The subcellular localization of Bcl10 is dynamically regulated in T cells. Bcl10 is found diffusely in the cytoplasm of most cells, and has also been found to exhibit nuclear trafficking [68, 70, 116]. However, upon TCR stimulation, Bcl10 is known to associate with binding partners in a pattern of cellular translocation that concentrates the protein in signaling structures that may aid in optimizing downstream signaling over the timeline of T cell activation. These signaling structures are primarily composed of CBM complex members CARMA1, MALT1, and Bcl10, and additional proteins that transiently interact with these proteins, such as RIP2 and TRAF6 [34]. Work from our group has previously described these enriched protein structures,

dubbed POLKADOTS [33, 34], as points at which Bcl10 and its binding partner MALT1 coalesce shortly after TCR stimulation. These enriched sites of signaling proteins then aggregate at a site beneath the SMAC. Over the course of Bcl10 coalescing and enriching in signaling structures, the NF-κB pathway is activated and initiates its own negative feedback loop, which includes the modification and subsequent degradation of the Bcl10 protein. As it is known that mutation of Bcl10, such as the CARD mutant G78R, can cause a dysregulation in the mechanism of Bcl10 translocation, I sought to determine if the Bcl10 N-terminal peptide may function in Bcl10 cellular trafficking. Specifically, I sought to determine if Bcl10 translocation could be affected by N-terminal peptide mutation during the first stage of Bcl10 trafficking after TCR stimulation, from diffuse cytoplasmic association to the concentrated POLKADOTS structure.

To observe the effect of Bcl10 N-terminal peptide mutants on Bcl10 translocation downstream of TCR stimulation, I used the mouse D10 T cell line. D10 cells were stably transduced with a pEhyg viral vector carrying a single given Bcl10 N-terminal peptide mutant. The mouse CH12 B cell line was used as the APC in these experiments, and was loaded either with no antigen or with the specific antigen for the D10 TCR, conalbumin (conA). D10 T cells and CH12 B cells were incubated for 20 minutes, the time point at which POLKADOTS formation appears to peak in T cells. Cell conjugates were then fixed, mounted, and visualized by fluorescence microscopy for the GFP tag on the Bcl10 constructs.

By using the Bcl10 mutant G78R [112] as a negative control and WT Bcl10 as a positive control of POLKADOTS formation in stimulated T cells, I was able to gauge the efficiency of Bcl10 N-terminal peptide mutants to form POLKADOTS [33, 34]. POLKADOTS structure (e.g. punctate or oligomeric-like), size, frequency, and subcellular location were assessed. Initial observations of WT Bcl10 with an N-terminal *vs.* C-terminal GFP tag is that the N-terminally

tagged construct (GFP-Bcl10), showed aberrant POLKADOTS formation (Figure 2B) despite having a WT protein sequence. The GFP-Bcl10 mutant, as previously described by other researchers [117], appears to localize to the cell membrane instead of forming the expected POLKADOTS structures. In addition to showing irregular cell membrane localization in stimulated cells, the GFP-Bcl10 construct showed protein aggregation unlike that seen in unstimulated WT Bcl10-GFP cells (Figure 2A).

The EED>AAA mutant had a specifically recurring phenotype where in unstimulated cells the mutant showed normal diffuse cellular behavior. However, upon cell stimulation, the mutant protein coalesced into POLKADOTS-like punctuate structures (Figures 2A and 2B). These EED>AAA punctuate structures were smaller in form and structure than WT Bcl10 POLKADOTS and showed none of the oligomeric-like qualities seen in WT POLKADOTS. As expected, the N-terminal peptide-only Bcl10 construct (1-12 only Bcl10) showed an inability to form any signaling structure either in stimulated or unstimulated T cells (Figures 2A and 2B), as this peptide does not have the sequence necessary to bind MALT1 or CARMA1 in order to form either POLKADOTS or the CBM complex. The mutants d2-12 and All-Ala showed similar impairment of Bcl10 translocation in stimulated T cells (Figure 2B), and remained diffusely cytoplasmic. Of note also is that the fluorescence intensity of GFP in these cells was extremely low and required much longer exposure of images to attain images of comparable brightness to those collected for WT Bcl10. The All-Ala mutant showed especially low GFP fluorescence, an indicator of poor Bcl10 protein expression in these cells.

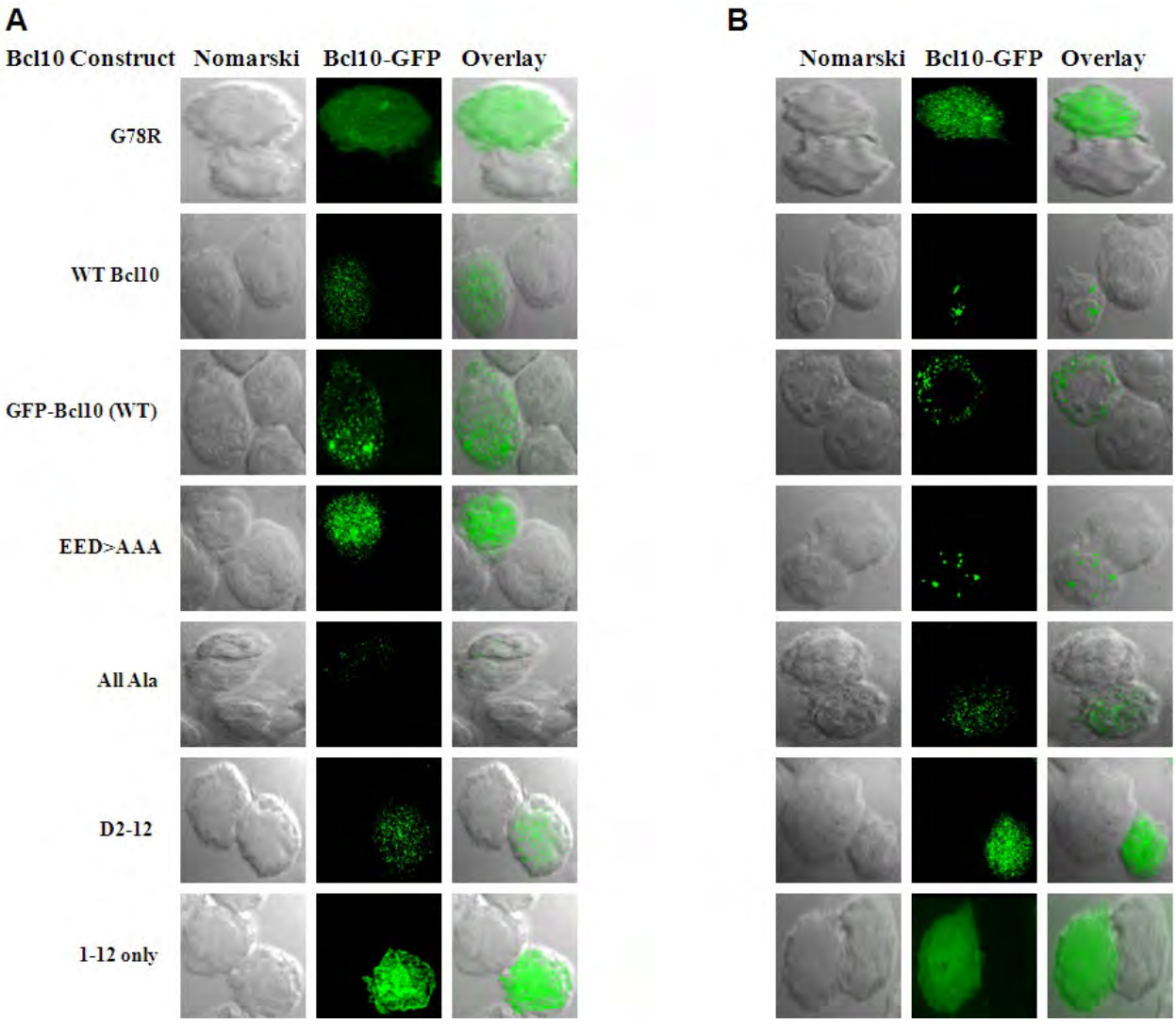


Figure 2: Mutation of the Bcl10 N-terminal peptide affects Bcl10 translocation upon T cell stimulation. Mouse D10 T cells were stably transduced with an N-terminal peptide-specific pEhyg Bcl10-GFP viral plasmid and then positively selected for by an antibiotic resistance gene included in the plasmid. D10 T cells were then incubated with (A) CH12 mouse B cells or (B) CH12 B cells that were loaded with their specific antigen (conalbumin). Conjugated T cells were fixed and mounted on slides at the 20 minute time point of incubation, when POLKADOTS are at their peak, and then analyzed using fluorescence microscopy to visualize each Bcl10-GFP construct. POLKADOTS of Bcl10 N-terminal peptide mutant T cells were compared to POLKADOTS of WT Bcl10 T cells only when the T cells were in conjugation with CH12 cells (to prevent any fluorescent artifacts not associated with TCR stimulation from being quantified). The length of excitation laser exposure for each Bcl10-GFP construct was dependent on how well the protein was expressed, with the d2-12 and All-Ala Bcl10 mutants being subjected to exposure times at least 20 times longer than WT Bcl10. GFP (green).

These experiments showed that mere steric hindrance of the N-terminal peptide by a GFP tag could severely disrupt Bcl10 protein translocation, impairing POLKADOTS formation and causing a higher concentration of Bcl10 translocation to the cell membrane than what is observed for C-terminally tagged Bcl10-GFP (Figure 2B) [33, 34, 117]. In addition, it appears the EED>AAA mutant could form POLKADOTS-like structures. However, these POLKADOTS differed in size, frequency, and intensity of those formed by WT Bcl10 protein. The complete absence or mutation of the N-terminal peptide (e.g. d2-12 or All-Ala mutants) causes Bcl10 to remain completely unresponsive to downstream TCR cellular stimulation. Most interesting perhaps is that although the All-Ala Bcl10 mutant is the equivalent size of a full-length Bcl10 protein, it showed no ability to form POLKADOTS. Also, downstream NF-κB activation by this mutant is inhibited in the very earliest stages of the NF-κB signaling cascade downstream of the TCR. These data regarding the role of the Bcl10 N-terminal peptide in Bcl10 subcellular localization to POLKADOTS after TCR stimulation suggest that this peptide sequence is critical for proper Bcl10 translocation.

Expression of Bcl10 N-terminal peptide mutants in D10 T cells is mutant sequence-specific (Figures 3A-C)

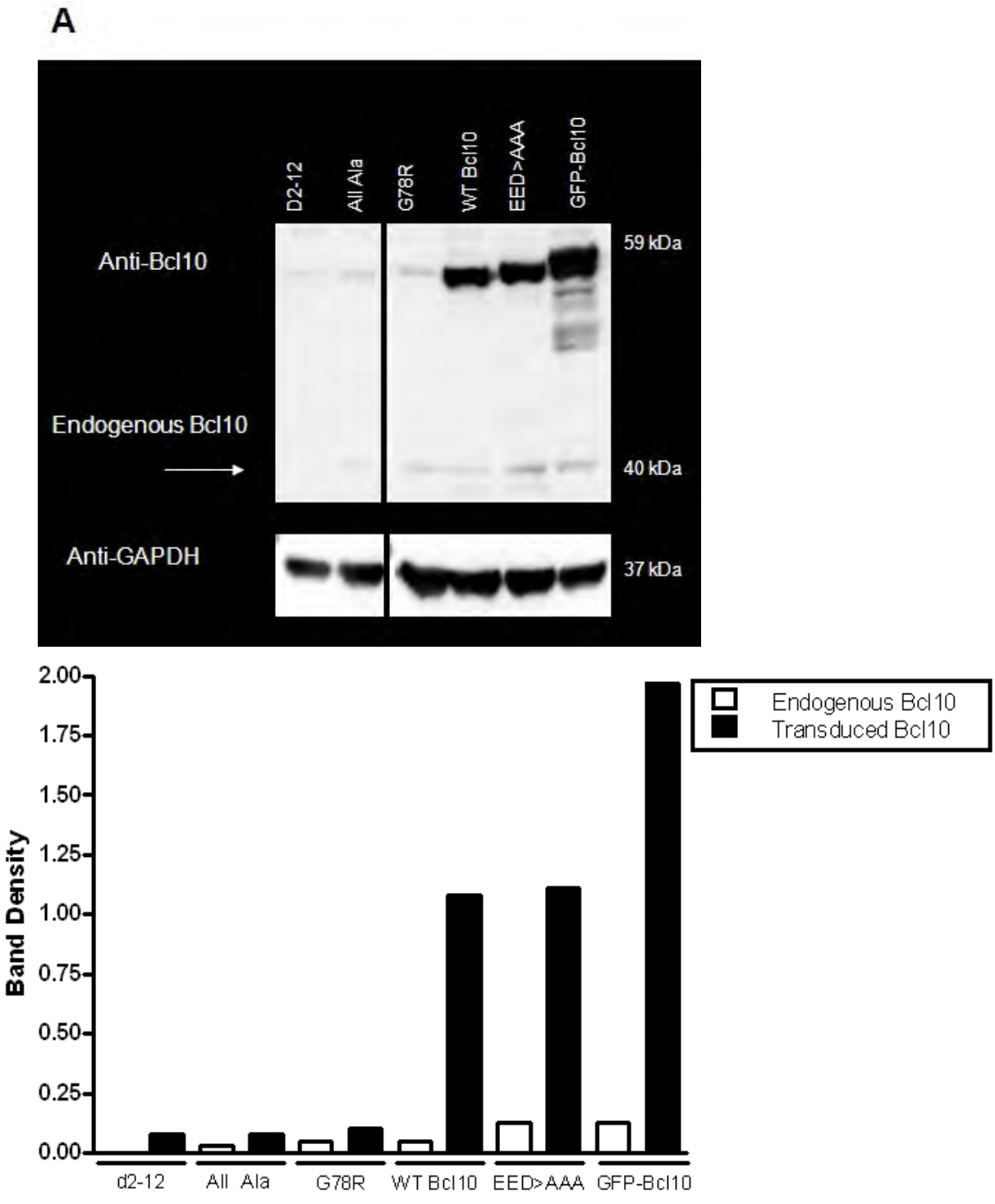
The results obtained in the NF-κB luciferase assays led me to believe that the Bcl10 N-terminal peptide mutants d2-12 Bcl10 and All-Ala Bcl10 were either incapable of activating the downstream NF-κB pathway or they were too poorly expressed by the cell to achieve the necessary level of downstream activation. Therefore, I sought to determine the relative levels of protein expression of select Bcl10 N-terminal peptide mutants to establish whether these proteins

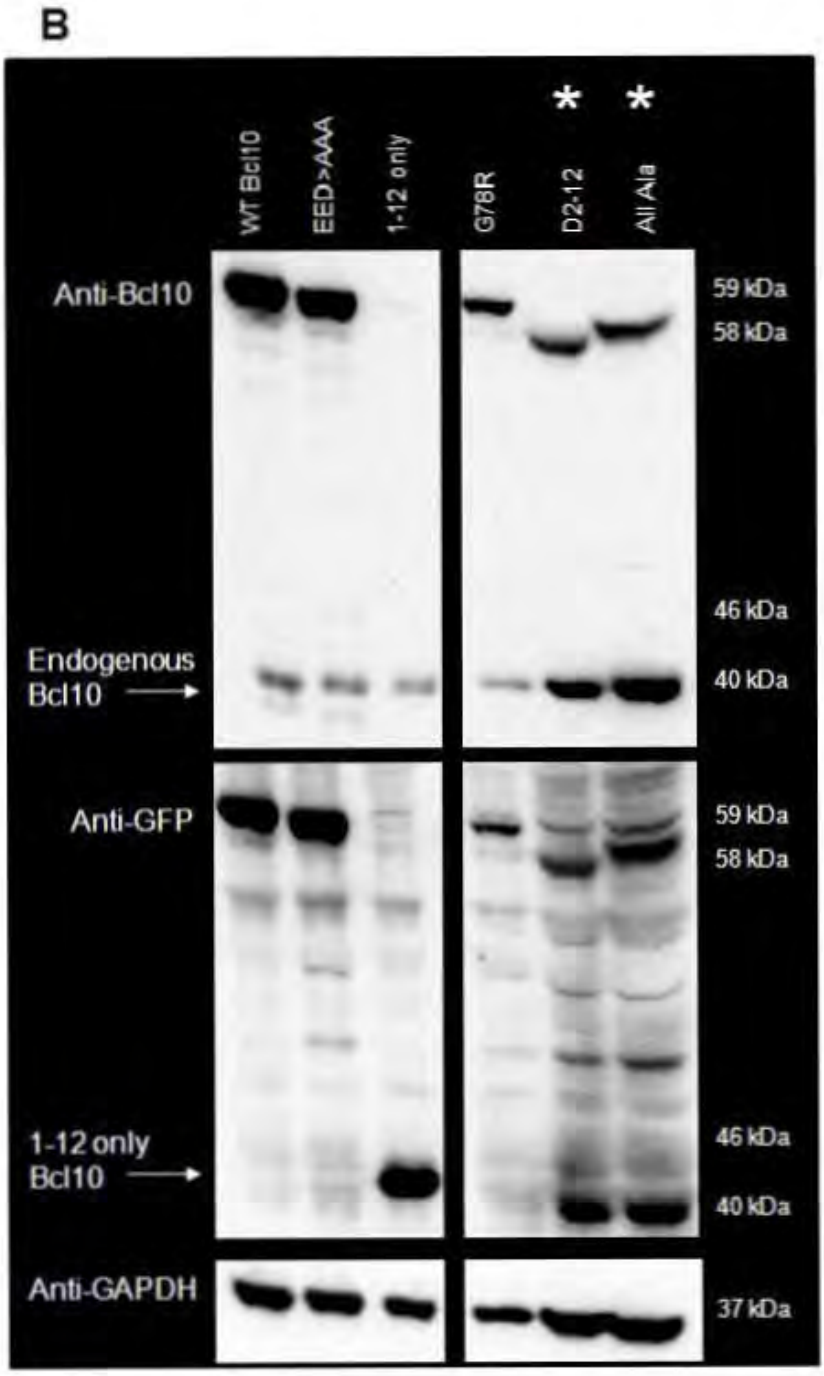
are expressed at a concentration comparable to WT Bcl10 and are truly incapable of activating the downstream NF-κB pathway, or were too poorly expressed to trigger the downstream NF-κB signaling cascade.

To obtain a better understanding of the steady-state levels of expression for various Bcl10 N-terminal peptide mutants, I used the D10 T cell lines, which stably expressed a pEhyg Bcl10 construct, as described above. The cells were collected as whole cell lysate (WCL) in samples of either 2×10^6 cells or 1×10^7 cells depending on observations on cellular protein expression over repeat experiments. Cellular lysates were run on an SDS-PAGE gel and then transferred to a nitrocellulose membrane for immunoblotting (western blot). Blots were probed with antibodies against Bcl10 or GFP to detect all Bcl10 constructs (the 1-12 only Bcl10 mutant can only be detected by its GFP tag). Blots were also probed for GAPDH as a loading control.

Western blots probed for Bcl10 protein, showed that some N-terminal peptide mutants exhibited protein concentrations far lower than that of WT Bcl10 (e.g. G78R Bcl10, d2-12 Bcl10, All-Ala Bcl10; Figures 3A-C), while other Bcl10 constructs were expressed at levels comparable to WT (e.g. 1-12 only Bcl10, EED>AAA Bcl10, S7AT9A Bcl10, S7DT9E Bcl10; Figures 3A-C). Notably, the d2-12 and All-Ala Bcl10 mutants were expressed at levels that were the lowest among the entire mutant sample set. As a result of these especially poor levels of protein expression, the d2-12 Bcl10 and All-Ala Bcl10 mutants were collected as WCL at cellular numbers five times that of Bcl10 mutant constructs that expressed protein at levels comparable to WT Bcl10 (1×10^7 cells *vs.* 2×10^6 cells per sample; Figure 3B). The data collected from these D10 T cell WCL western blots clearly showed that not all of the Bcl10 N-terminal peptide mutants expressed protein at steady-state levels comparable to those of WT Bcl10. Although many Bcl10 N-terminal peptide mutants do show levels of protein expression

similar to WT Bcl10, the mutants that were unable to activate NF-κB in the NF-κB luciferase assays (Figure 1) also exhibited an approximately five fold reduction in protein expression relative to WT Bcl10. These results may be indicative of a high turnover rate of the d2-12 Bcl10 and All-Ala Bcl10 mutants that may impair them from efficiently activating downstream NF-κB.

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Anti-GFP
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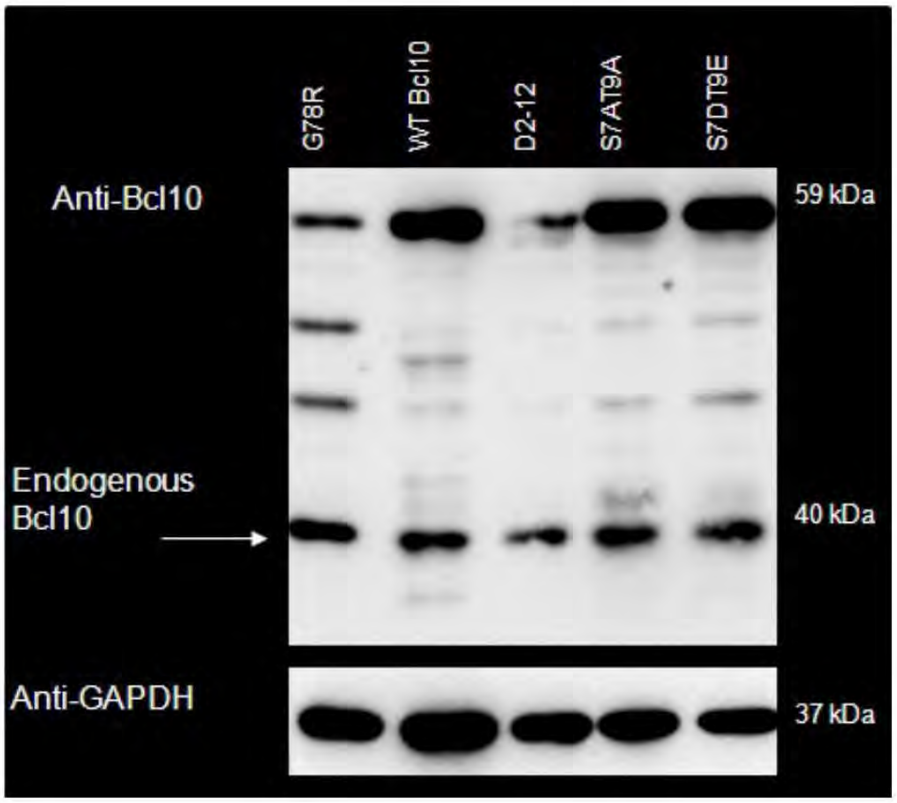
C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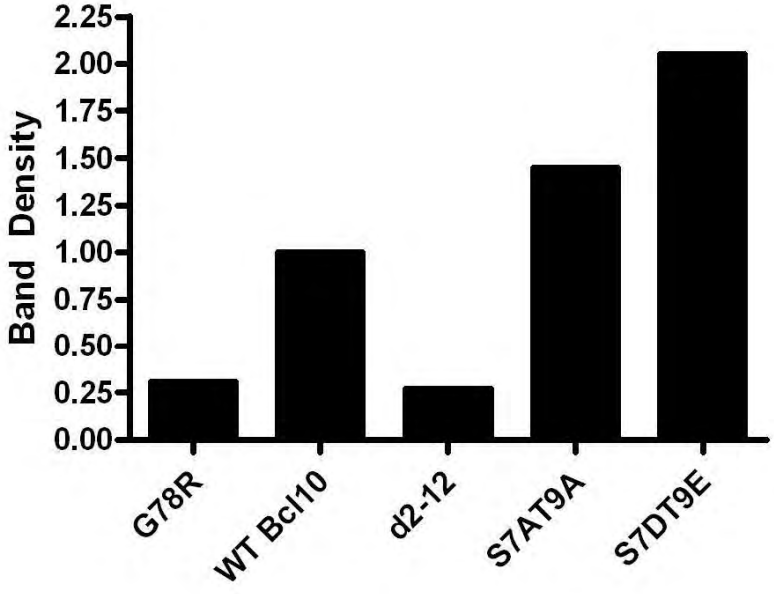
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1.25
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0.50
0.25
0.00
G78R
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d2-12
S7AT9A
S7DT9E

Figure 3: D10 T cell expression of Bcl10 is N-terminal peptide sequence-specific. (A-C) D10 T cells stably transduced with a single Bcl10-GFP construct were collected as whole cell lysate in samples of 2 x 10^6 cells. Cellular lysates were run on an SDS-PAGE gel and transferred to a nitrocellulose membrane for immunoblotting. Blots were probed with antibodies against Bcl10 or GFP to detect all Bcl10 constructs (1-12 only Bcl10 can only be detected by its GFP tag). Blots were probed for GAPDH as a loading control. (*) d2-12 and All Ala each have 1 x 10^7 cells per sample.

Bcl10 binding partners show some reliance on the N-terminal peptide for protein-protein interactions (Figures 4A-E)

The Bcl10 protein has several different functions in multiple cellular pathways. As a protein ubiquitously expressed in every cell type, Bcl10 is critical for several distinct NF-κB activation pathways. The CARD domain of Bcl10 makes it an important member of a group of proteins that interact in an oligomerized fashion with fellow CARD proteins. The Bcl10 CARD domain is also important in the adaptor function of Bcl10. In addition, the Bcl10 serine/threonine-rich C-terminal domain becomes highly phosphorylated in response to TCR stimulation, although the significance of these phosphorylations remains unclear [55]. To date, the function of the N-terminal peptide of Bcl10 has remained largely unexplored. I thus performed experiments to determine whether the N-terminal domain has an important role as a target of post-translational modification and/or as a site of binding to other proteins.

To observe the importance of the N-terminal peptide with respect to key Bcl10 binding partners, I performed co-immunoprecipitation (co-IP) of Bcl10 N-terminal peptide mutants with binding partners to observe how well each mutant can bind when the N-terminal peptide is mutated, relative to WT Bcl10. HEK-293T cells were transiently transfected with a pcDNA3 3xHA Bcl10 construct in conjunction with one binding partner of interest cloned into pcDNA3 (or pEZeo, for the vE10 plasmid) with a 3xFLAG tag. Proteins of the CBM complex were examined, and I also assessed the effect of mutations on Bcl10 homodimerization. The co-IPs

thus examined Bcl10 mutant constructs co-transfected with WT Bcl10, CARMA1, CARMA3, MALT1, and vE10. Cellular lysates were collected on Day 2 after transfection and split into two pools, so that approximately 35% of the total cell sample would be used as whole cell lysate (WCL), while the remaining cells were used for the co-IP assay. Co-IP cell lysates were subjected to lysis in a buffer containing protease and phosphatase inhibitors to maintain protein integrity, and then pre-cleared with Protein G sepharose beads. Pre-cleared lysates were then incubated with an anti-FLAG antibody to directly pull-down FLAG-tagged WT Bcl10, CARMA1, CARMA3, vE10, or MALT1 complexed with the co-transfected HA-tagged Bcl10 construct. Protein complexes were eluted using Protein G beads and the lysates were then triple washed with buffer to remove extraneous proteins. Precipitated protein complexes and WCLs were run on an SDS-PAGE gel and immunoblotted for Bcl10 (using an anti-HA antibody), the given binding partner (WT Bcl10, CARMA1, CARMA3, MALT1, or vE10; using a distinct anti-FLAG antibody), and also a loading control.

I observed an array of binding affinities amongst the Bcl10 binding partners and the N-terminal peptide mutants (Figure 4A-E). For co-IP of CARMA1-Bcl10 complexes, I used Bcl10 mutant G78R, which has a CARD mutation that prevents proper CARD folding, as a negative control [112], and WT Bcl10 as a positive control. As seen in Figure 4A, relative to WT Bcl10, no N-terminal peptide mutant could efficiently complex with CARMA1. The d2-12 mutant of Bcl10 especially showed no perceivable binding to CARMA1. Additionally, the EED>AAA Bcl10 mutant showed significantly impaired binding to CARMA1. It is important to note however, that the d2-12 mutant was expressed poorly, as indicated by the WCL samples. Thus, the observed lack of binding of d2-12 to CARMA1 may reflect, in part, the lower concentration of d2-12 protein in the cells, relative to other Bcl10 constructs. The lower concentration of d2-

12 Bcl10 protein expression, and other Bcl10 constructs showing poor protein expression, was compensated for by increasing the concentration of DNA transfected for those constructs.

The MALT1-Bcl10 co-IP (Figure 4B) was illustrative of the N-terminal peptide's role in Bcl10 interactions with partner proteins. Similar to what I observed in the luciferase assays (Figure 1), correlating the length of the N-terminal peptide to NF-κB activation efficiency, the MALT1-Bcl10 interaction efficiency depends on the length of the N-terminal peptide. Specifically, using the Bcl10 mutant dMALT1 (in which an essential portion of the MALT1 binding site has been deleted) [30] as a negative control, I observed that the longer the deletion of the N-terminal peptide (e.g. d2-4 mutant *vs.* d2-12 mutant), the poorer the MALT1-Bcl10 interaction. Similar to the CARMA1 co-IP, I noted that the EED>AAA mutant also showed a clear reduction in binding to MALT1, relative to WT Bcl10.

I also assessed the ability of WT Bcl10 to homodimerize with itself when the N-terminal peptide is mutated. As expected, WT Bcl10 showed strong binding and G78R Bcl10 showed weak binding, in co-IPs with WT Bcl10. Although Bcl10-Bcl10 complexes did not form for the d2-12 mutant, the expression level was consistently too low for meaningful comparison to the other constructs (data not shown). However, co-IP of the EED>AAA mutant with WT Bcl10 showed impaired binding capacity, analogous to that seen in the CARMA1 co-IP (Figure 4A and 4C).

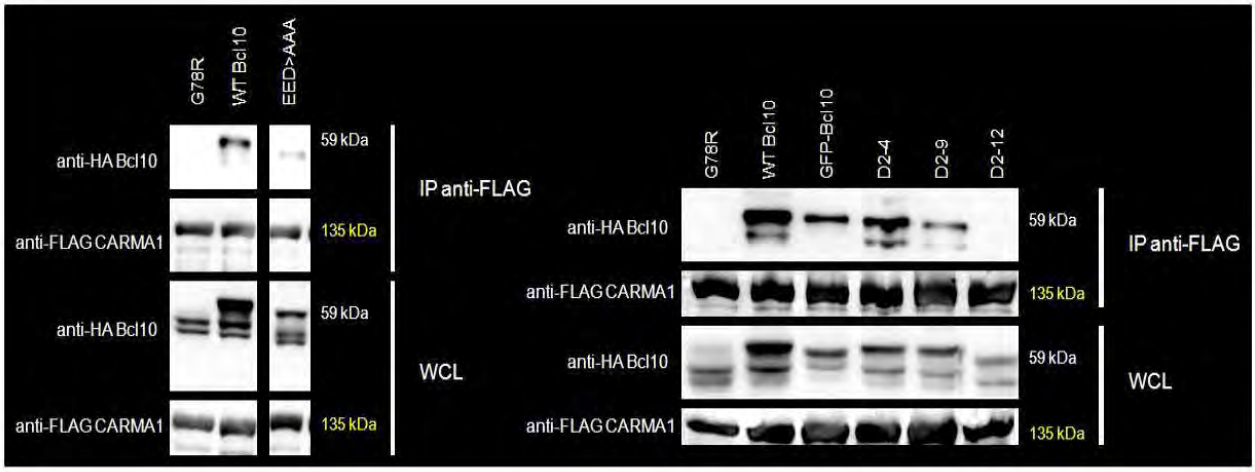
Additionally, I examined the propensity of vE10, another CARD family member, to co-IP Bcl10 N-terminal peptide mutants (Figure 4D). The equine herpes viral protein E10 (vE10) is known to be associated with a specific pathogenesis in the infected lymphoid cells of horses [76]. How the vE10 protein functions in other cell types has been less defined to date. As vE10 is a CARD protein and interacts with Bcl10 through its CARD domain in a method similar to that of

CARMA1 with Bcl10, I expected that certain N-terminal peptide mutants would behave similarly to what was seen in Figure 4A, with little to no protein binding between these more extreme N-terminal peptide mutants and the vE10 binding partner. The purpose of this Bcl10 and vE10 co-IP assay was to determine the binding strength of some N-terminal peptide mutants with vE10, relative to WT Bcl10.

As Bcl10 and vE10 binding is dependent on CARD domain interaction, the Bcl10 CARD mutant G78R was used as a negative control. Using two different protein concentrations, I observed that the G78R Bcl10 negative control does not co-precipitate with vE10, as compared to the WT Bcl10 control, which binds vE10 well over two different transfected DNA concentrations (Figure 4D). The EED>AAA Bcl10 mutant exhibits some binding to vE10 however, it is much diminished in comparison to WT Bcl10 co-IP (Figure 4D), similar to the co-IP phenotype observed in the CARMA1 co-IP immunoblots (Figure 4A). Thus, mutation of the Bcl10 N-terminal peptide impairs the interaction between Bcl10 and vE10.

An analogous CARD protein to CARMA1 in the NF-κB pathway of non-leukocytes is CARMA3. When Bcl10 N-terminal peptide mutants were co-transfected with CARMA3 I found that they did not follow the results of those binding capacities I observed between CARMA1 and Bcl10 N-terminal peptide constructs (Figures 4A and 4E). One point of similarity between the two CARD proteins was that CARMA3 showed an impairment in binding EED>AAA Bcl10. However, unlike many of the other tested binding partners, including CARMA1, CARMA3 could bind to d2-12 Bcl10, even though there was a diminished binding affinity when compared to WT and EED>AAA Bcl10 (Figure 4E).

A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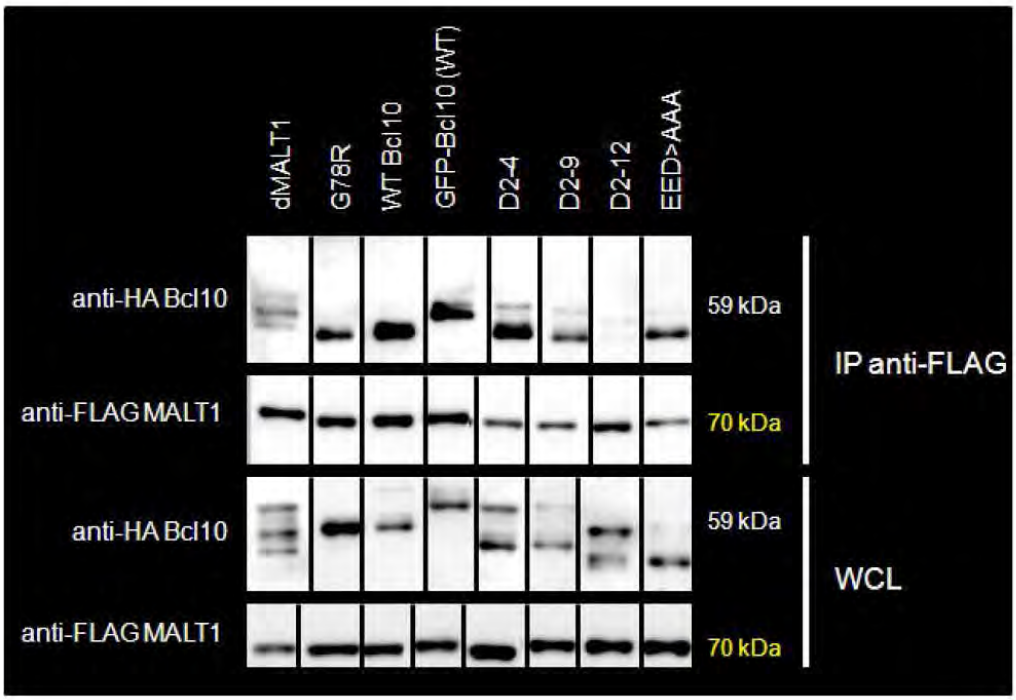
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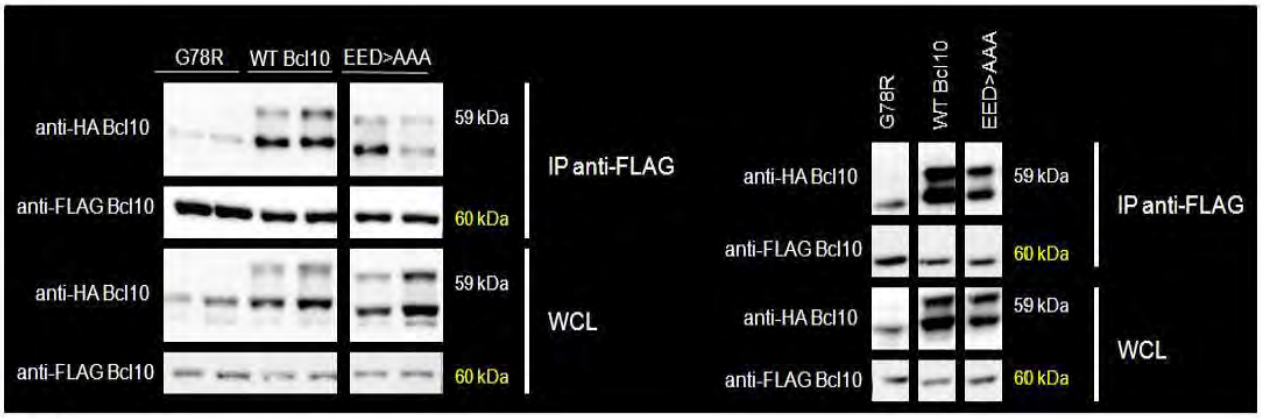
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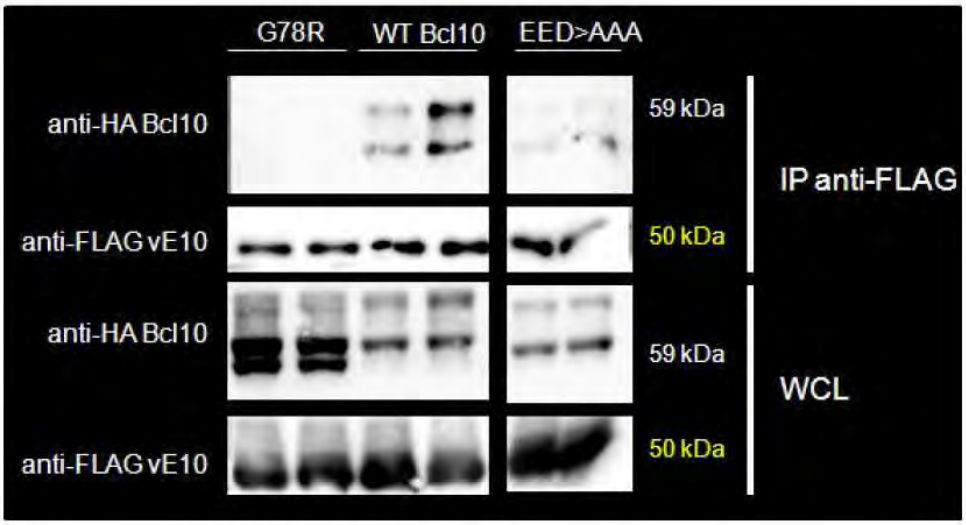
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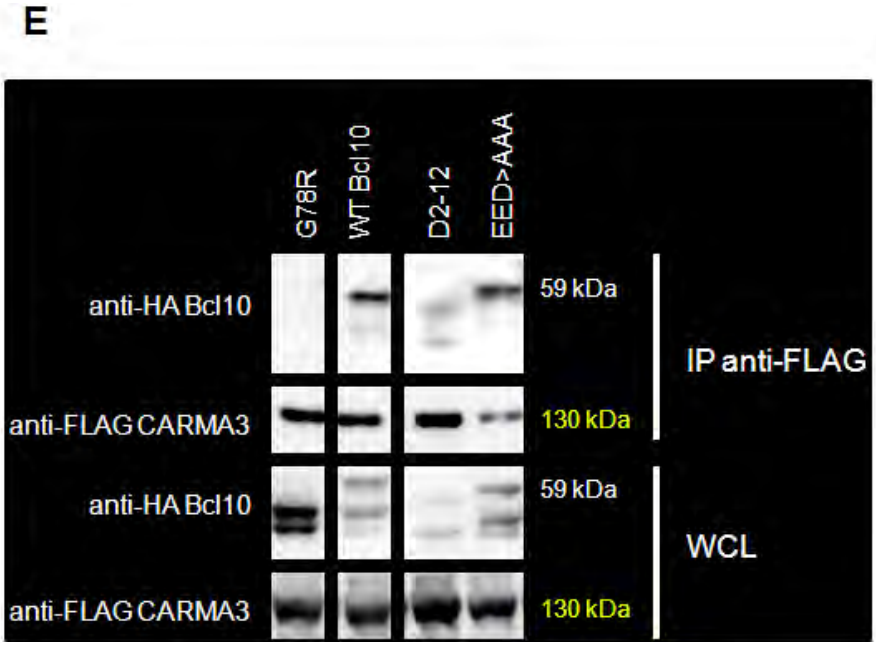


Figure 4: Co-immunoprecipitation of Bcl10 binding partners is affected by the N-terminal peptide sequence. HEK-293T cells were transiently transfected with a pcDNA3 3xHA-Bcl10 construct in conjunction with one binding partner of interest cloned into pcDNA3 (or pEZeo for vE10) with a 3xFLAG tag. Cellular lysates were collected on Day 2 after transfection and split into two pools, with approximately 35% of the total cell sample used as whole cell lysate (WCL), and the remaining used for co-immunoprecipitation (co-IP) assay. Co-IP cell lysates were subjected to lysis in a buffer containing protease and phosphatase inhibitors, and then pre-cleared with Protein G sepharose beads. Pre-cleared lysates were then incubated with an anti-FLAG antibody to pull-down FLAG-tagged WT Bcl10, CARMA1, CARMA3, vE10, or MALT1 complexed with their co-transfected HA-tagged Bcl10 construct. Protein complexes were eluted using Protein G beads and the lysates were then triple washed with buffer to remove extraneous proteins. Pure protein complexes and WCLs were run on an SDS-PAGE gel and immunoblotted for Bcl10 (using an anti-HA antibody), the given binding partner ((A) CARMA1, (B) MALT1, (C) WT Bcl10, (D) vE10, or (E) CARMA3; using a distinct anti-FLAG antibody), and also a loading control.

Although this analysis was somewhat hampered by great difficulty in expressing Bcl10 mutant constructs at similar levels within transfected cells, important conclusions can be made. Overall, I have observed that the Bcl10 N-terminal peptide is required for efficient Bcl10 binding to many (but not all) partner proteins. Thus, the N-terminal peptide likely plays a supporting role in the functional interaction between the Bcl10 CARD domain and several partner proteins. Moreover, as demonstrated by MALT1, which binds to the C-terminus of the Bcl10 CARD and a minimal domain downstream of the CARD, it appears that alteration of the Bcl10 N-terminal

peptide can affect binding properties far downstream of the mutant sequence, even in the context of the missense mutation, EED>AAA. Finally, Bcl10 binding to CARMA3 was apparently not strongly affected by the d2-12 and EED>AAA mutations. These data therefore strongly suggests that the d2-12 and EED>AAA mutations do not simply result in gross misfolding of the CARD (as with the G78R mutation). This conclusion is supported by several independent pieces of data (see below).

Aim 2

Determine the function of the Bcl10 N-terminal peptide with respect to Bcl10 expression, stability, and modification.

The Bcl10 N-terminal peptide mutants are not grossly misfolded, as demonstrated by retaining binding to the Bcl10 homolog, vE10 (Figures 5A and 5B)

The equine herpes virus (EHV) E10 (vE10) protein is a CARD family member and close homolog of the somewhat smaller Bcl10 protein. Previous studies of vE10 have identified its ability to recruit Bcl10 to the cell membrane and initiate downstream NF-κB activation [77, 80]. It has been previously noted that vE10 binds to cellular Bcl10, in essence hijacking Bcl10 from its defined mechanistic functions, re-localizing Bcl10 to the cell membrane via vE10's C-terminal geranyl-geranylation domain. By an uncertain mechanism, membrane localization of Bcl10 leads to uncontrolled NF-κB activation, which contributes to chronic pharyngitis that is associated with EHV-infected lymphoid cells [76]. vE10 binds to Bcl10 through its CARD domain, an interaction similar to that of Bcl10-Bcl10 homodimerization and Bcl10 interaction

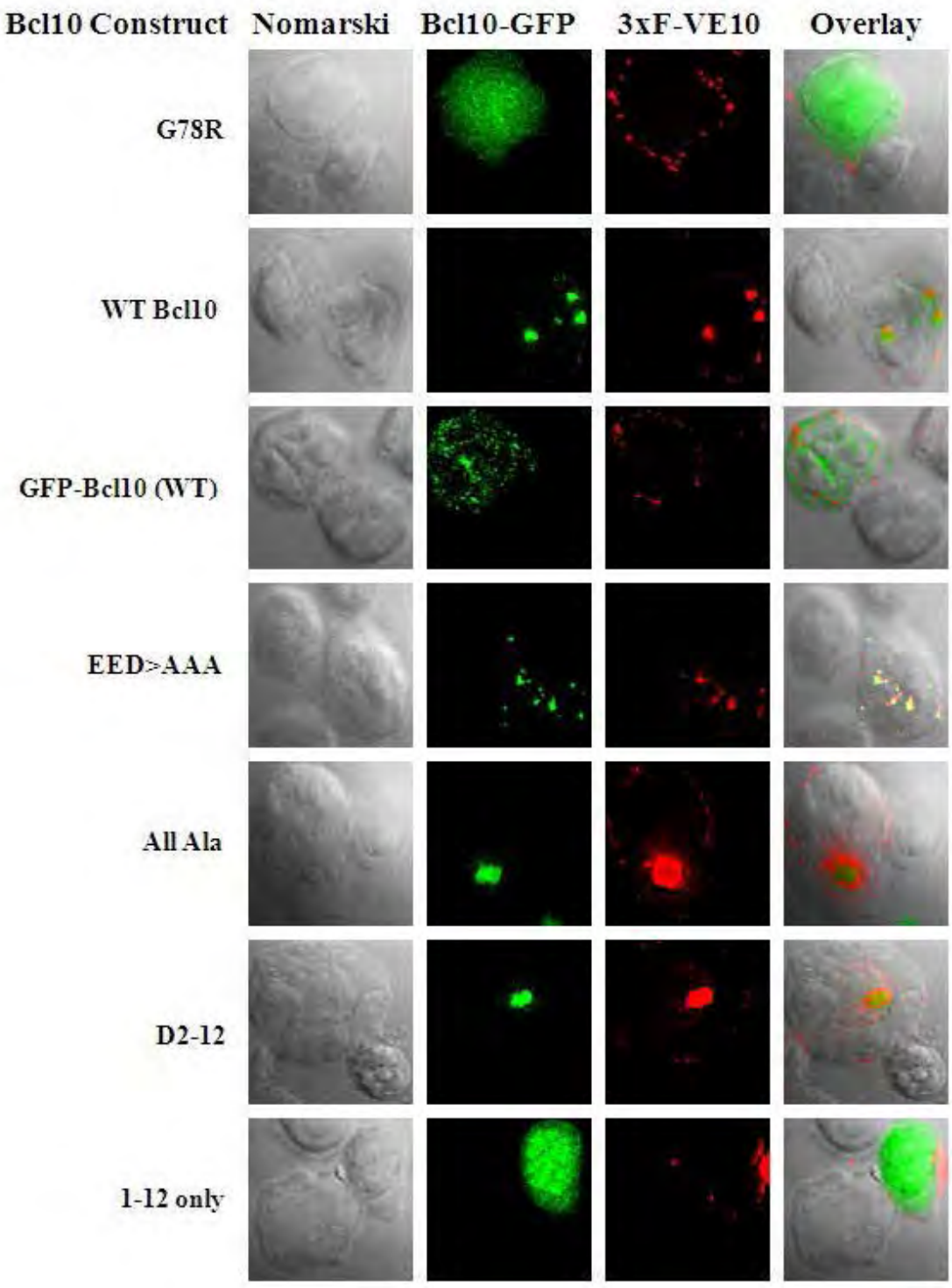
with the CARMA family of proteins, such as CARMA1, in immune cells. I sought to determine if vE10 could interact with and re-localize Bcl10 N-terminal peptide mutants. Data from preceding experiments brought me to two possible hypotheses: the first is that d2-12 Bcl10 and a few other N-terminal peptide mutants are poor activators, but are structurally intact; or the second, which is that d2-12 Bcl10 and a few other N-terminal peptide mutants are somehow required for proper folding and/or stabilization of the Bcl10 CARD. These experiments with vE10 were thus conducted to assess whether vE10 can bind to each Bcl10 N-terminal peptide mutant (especially d2-12 Bcl10), to determine if the CARD of these Bcl10 mutants is intact.

Using a method previously described in Aim 1, I transduced D10 T cells that already stably expressed a Bcl10 construct with a plasmid encoding pEZeo 3xFLAG-vE10. The next day, cells were incubated for 20 minutes with CH12 B cells that had been incubated overnight with or without 250 μM of their specific antigen, conalbumin. Cells were then fixed, permeabilized, and stained with anti-FLAG to detect the vE10 3xFLAG tag (using an Alexa647-labeled secondary antibody). Cells were then mounted on slides and imaged by fluorescent microscopy. Using this technique, I sought to observe the localization behavior of Bcl10 in the presence of vE10 in both unstimulated T cells and in T cells undergoing TCR stimulation via conjugated, antigen-loaded B cells. As previously discussed, Bcl10 in unstimulated cells remains diffusely cytoplasmic, while Bcl10 at 20 minutes post-TCR stimulation shows localization to POLKADOTS [33, 34]. Also, Bcl10 with vE10 co-expression shows aberrant protein localization to the cell membrane [80]. However, it is important to note that POLKADOTS formation in T cells expressing the vE10 protein has never been tested before.

As has been observed by Thome *et al* [80], in unstimulated cells, the vE10 protein binds to, hyperphosphorylates, and re-localizes cytoplasmic Bcl10 to the cell membrane. Using the

Bcl10 CARD folding mutant, G78R [112], as a negative control and WT Bcl10 as a positive control, I compared the co-localization patterns of specific Bcl10 N-terminal peptide mutants (Figures 5A and 5B). In both unstimulated and stimulated T cells, I observed that the G78R Bcl10 mutant failed to co-localize with vE10 at the cell membrane, and instead remained diffusely cytoplasmic (Figures 5A and 5B). As expected, the positive control for vE10 co-localization, WT Bcl10, showed redistribution to the cellular membrane in both stimulated and unstimulated cells (Figures 5A and 5B). The construct encoding the N-terminal peptide alone (1-12 only Bcl10) could not co-localize with vE10 because it did not have the CARD domain necessary for binding to the homologous vE10 CARD domain. This construct thus remained diffusely cytoplasmic, despite vE10 localization to the cell membrane (Figures 5A and 5B). In contrast, the Bcl10 protein variant with N-terminal GFP (GFP-Bcl10) localized to the cell membrane, regardless of cell stimulation (Figures 5A and 5B). Thus, GFP-Bcl10 efficiently binds to vE10 when both proteins are expressed in D10 cells. The EED>AAA Bcl10 mutant also showed ready vE10 co-localization similar to that observed with WT Bcl10 in both stimulated and unstimulated T cells (Figures 5A and 5B). Most important were the co-localization results from the d2-12 Bcl10 and All-Ala Bcl10 mutants. Although these mutants were unable to form POLKADOTS in response to TCR stimulation (Figure 2), each of these mutants co-localized with vE10 and showed redistribution to the cell membrane in both stimulated and unstimulated T cells (Figures 5A and 5B). Lastly, for every Bcl10 construct that did co-localize with vE10 at the cell membrane it is important to note that vE10 re-localization of Bcl10 superseded POLKADOTS formation in stimulated T cells, showing a dominant ability of vE10 to bind to and traffic Bcl10.

A


Bcl10 Construct
Nomarski
Bcl10-GFP
3xF-VE10
Overlay
G78R
WT Bcl10
GFP-Bcl10 (WT)
EED>AAA
All Ala
D2-12
1-12 only

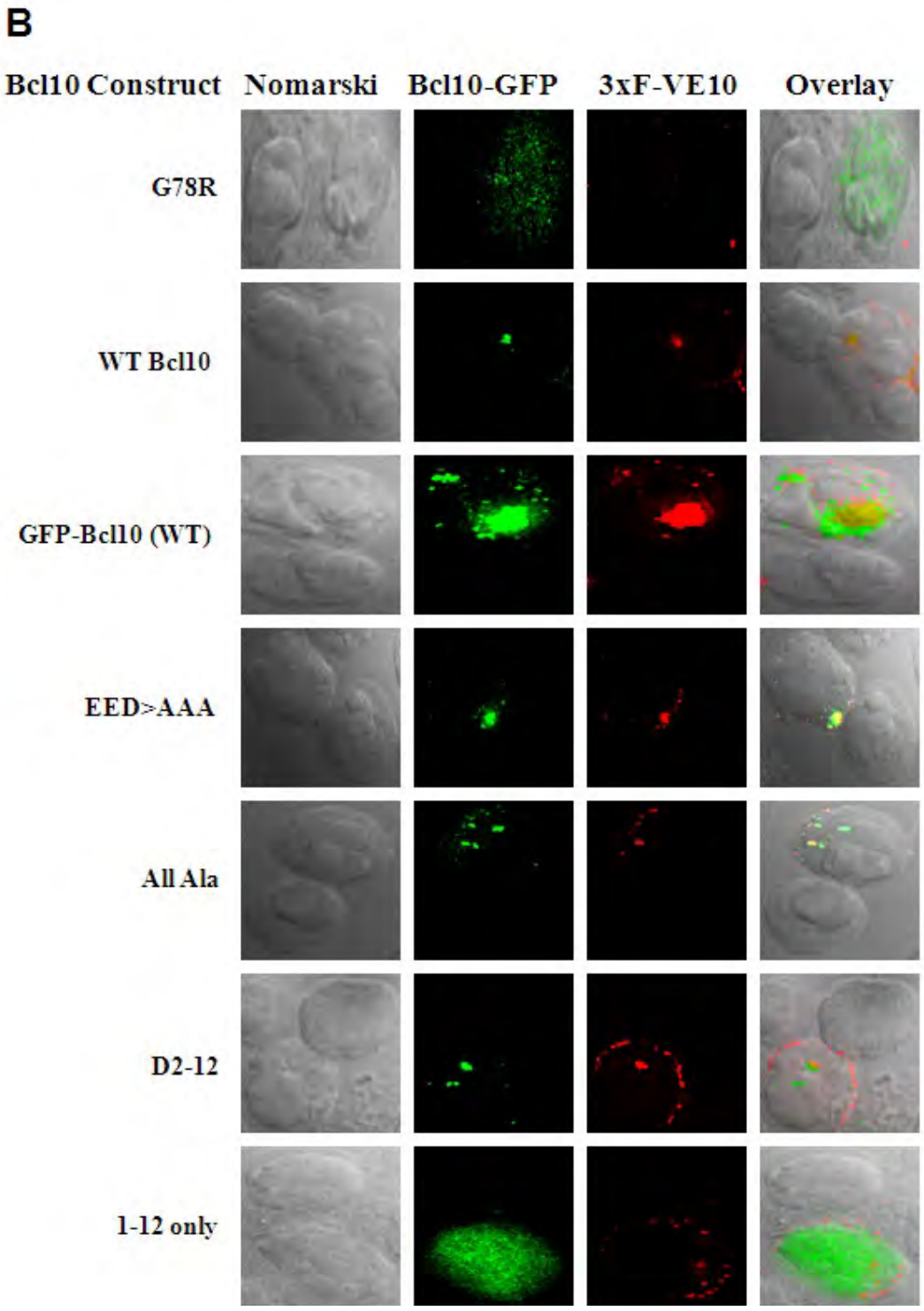


Figure 5: Bcl10 N-terminal peptide mutants relocalize with the CARD protein vE10. D10 T cells were co-transduced with one pEhyg Bcl10 construct and pEZeo 3xFLAG-vE10. The next day, the D10 T cells were incubated with (A) CH12 B cells or (B) CH12 B cells loaded with their specific antigen (conalbumin) for 20 minutes. Cells were then fixed, permeabilized, and stained with an anti-FLAG primary antibody to detect the vE10 3xFLAG tag and an Alexa647-labeled anti-FLAG secondary antibody. Cells were then mounted on slides and imaged by fluorescent microscopy. GFP (green),

The Bcl10 N-terminal peptide mutant data showed that relative to the Bcl10 CARD negative control (G78R Bcl10), the N-terminal peptide mutants can still efficiently bind to and localize with vE10. Thus, the Bcl10 N-terminal peptide is not required for all types of Bcl10 protein-protein interaction. The impaired binding seen with the co-IP experiments with CARMA1, MALT1, and WT Bcl10, possibly reflects a contributing role of the N-terminal peptide to protein interactions between Bcl10 and several binding partners. As it has been previously demonstrated that Bcl10 binding to vE10 occurs via a CARD-CARD interaction [80], this data strongly suggest the N-terminal peptide controls Bcl10-partner protein interactions through a mechanism other than maintaining the conformational stability of the Bcl10 CARD.

Bcl10 protein stability is reduced when the N-terminal peptide is mutated (Figures 6A-B)

The Bcl10 protein is ubiquitously expressed in all cell types. In T cells, Bcl10 undergoes regimented degradation following TCR stimulation, limiting the extent of NF-κB activation. However, the Bcl10 protein in un-stimulated cells is maintained at a stable level. Because this data suggested that the N-terminal peptide influences steady-state turn-over of Bcl10, I performed experiments to measure the rate of steady-state Bcl10 degradation.

Using cycloheximide (CHX) to inhibit protein translation, I examined two different cell types to determine the steady-state degradation rate of several Bcl10 N-terminal peptide constructs over a time course. Using D10 T cells stably transduced with a given Bcl10 construct, I either treated cells with DMSO (vehicle control) or with 50 μg of CHX for the time points stated. Cells were then collected as whole cell lysate and run on an SDS-PAGE gel. The gel

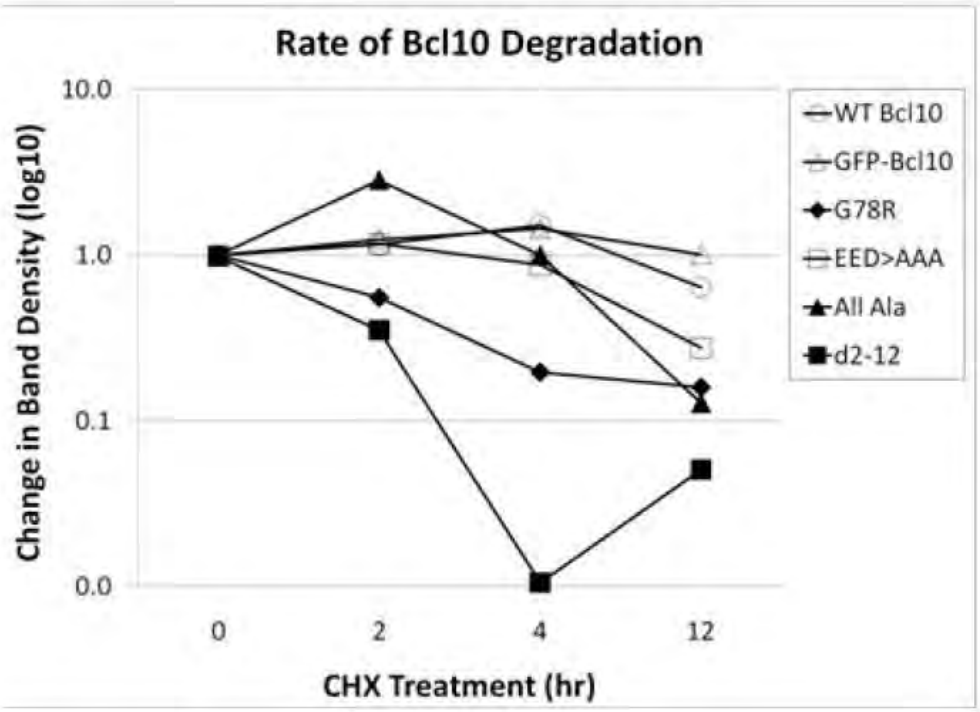
was then transferred to a nitrocellulose membrane for western blotting where an anti-Bcl10 antibody was used to probe the blot for total Bcl10 protein. Blots were stripped and re-probed for the loading control GAPDH and for the positive control, cyclin D3, which is an important cell cycle regulator that exhibits a rapid rate of proteasome-dependent turnover. In addition to D10 T cells, I used HEK-293T cells to quantify the effect of CHX treatment on Bcl10 turn-over. HEK cells were transiently transfected with a given pcDNA3 3xHA-Bcl10 construct. On Day 2 post-transfection, cells were treated in a similar manner to that of D10 T cells, with vehicle or 50 μg of CHX for the time points given. HEK whole cell lysates were prepared and subjected to western blotting, in an analogous manner to that stated for the D10 T cell whole cell lysates. Using the expression levels of GAPDH as an indicator of cell viability, and the expression levels of cyclin D3 as an indicator of CHX effectiveness, I was able to quantify the kinetics of Bcl10 protein degradation for each N-terminal peptide mutant tested, relative to WT Bcl10 protein concentration.

As I previously observed, certain Bcl10 N-terminal peptide mutants did not express as well as WT Bcl10. However, the goal of these CHX assays was to determine if these Bcl10 N-terminal peptide mutants degrade at a rate that was slower, equal to, or faster than the rate exhibited by WT Bcl10. I observed that in cells treated with CHX, relative to WT, the N-terminal peptide mutants degrade at a rate much faster than that of WT Bcl10 (Figures 6A-B). The rate of fastest Bcl10 protein turnover is exhibited by the d2-12 and All-Ala mutants, with little to no Bcl10 protein being detectable after 4 hr of CHX treatment (Figures 6A-B). In D10 T cells (Figure 6A) both WT Bcl10 and GFP-Bcl10 were very stable over a period of at least 12 hr. The CHX assay conducted with transfected HEK cells allowed me to define which Bcl10 isoforms were degraded over time (Figure 6B). Figure 6B shows that over the time of CHX

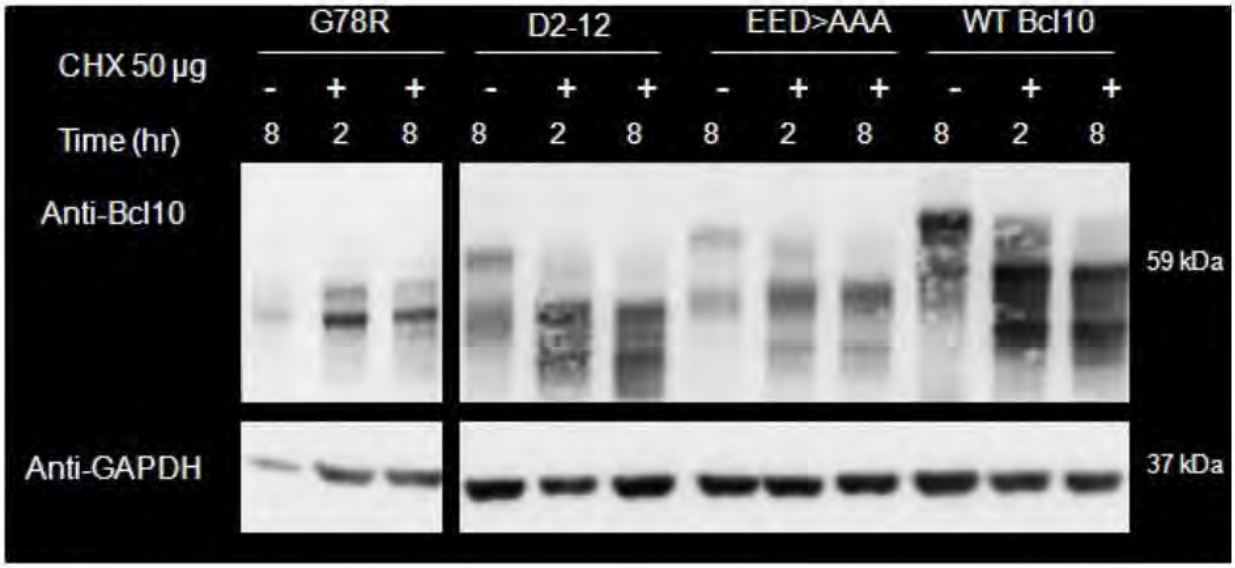
treatment, the top Bcl10 band (hyperphosphorylated Bcl10) [33] diminishes over time, while a lower molecular weight form of Bcl10 appears with similar kinetics. This pattern is observed for all Bcl10 isoforms, except G78R, which largely lacks this hyperphosphorylated form of Bcl10. I observed that, like WT Bcl10, the d2-12 and EED>AAA Bcl10 mutants exhibited a steady degradation of their highest (phosphorylated) isoforms. Importantly, degradation of the hyperphosphorylated d2-12 Bcl10 band occurred at a faster rate than observed with WT Bcl10. By contrast, the un- and under-phosphorylated (middle) Bcl10 isoforms were stable over time for all Bcl10 constructs. However, the lower molecular weight (cleaved) isoforms increased substantially over time, with kinetics closely matching the kinetics of the loss of the phosphorylated band. These results suggest that the hyper-phosphorylated form of Bcl10 is vulnerable to rapid proteolytic cleavage. Furthermore, the N-terminal peptide mutants (e.g. d2-12) may be more susceptible to this cleavage and degradation than WT Bcl10. Overall, the results of CHX treatment of cells indicate the rate of Bcl10 protein degradation is enhanced by mutation of the N-terminal peptide, relative to WT. The more rapid protein degradation kinetics exhibited by the d2-12 and All-Ala Bcl10 mutants may suggest that the N-terminal peptide directly protects Bcl10 from hyperphosphorylation-mediated degradation. Alternatively, these N-terminal peptide mutants may disrupt a binding site required for stable interaction with one or more Bcl10 binding partners. By this model, the unbound hyperphosphorylated form of Bcl10 is more vulnerable to proteolytic degradation.

A


G78R
D2-12
All Ala
EED>AAA
WT Bcl10
GFP-Bcl10 (WT)
Hours
0 2 4 12 0 2 4 12 0 2 4 12 0 2 4 12 0 2 4 12 0 2 4 12
CHX
- + + + - + + + - + + + - + + + - + + + - + + +
anti-Bcl10
59 kDa
anti-Cyclin D3
anti-GAPDH
37 kDa


Rate of Bcl10 Degradation
Change in Band Density (log10)
10.0
1.0
0.1
0.0
0
2
4
12
CHX Treatment (hr)
WT Bcl10
GFP-Bcl10
G78R
EED>AAA
All Ala
d2-12

B


G78R
D2-12
EED>AAA
WT Bcl10
CHX 50 µg
- + + - + + - + + - + +
Time (hr)
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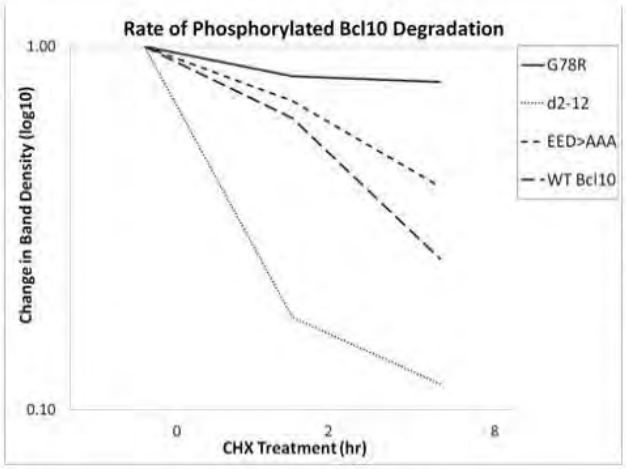
59 kDa
Anti-GAPDH
37 kDa


Rate of Phosphorylated Bcl10 Degradation
Change in Band Density (log10)
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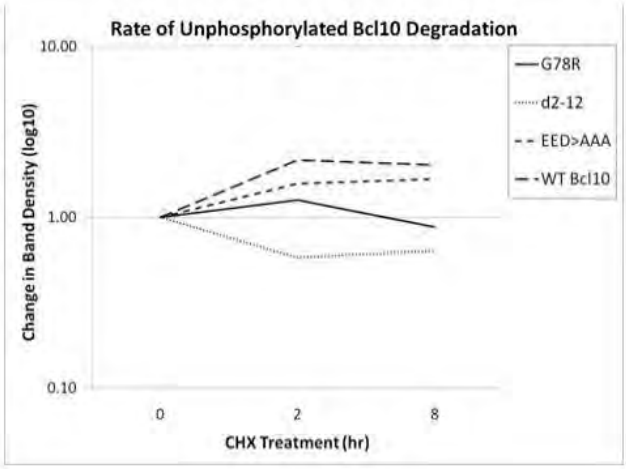
0.10
0
2
8
CHX Treatment (hr)
G78R
d2-12
EED>AAA
WT Bcl10


Rate of Unphosphorylated Bcl10 Degradation
Change in Band Density (log10)
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1.00
0.10
0
2
8
CHX Treatment (hr)
G78R
d2-12
EED>AAA
WT Bcl10

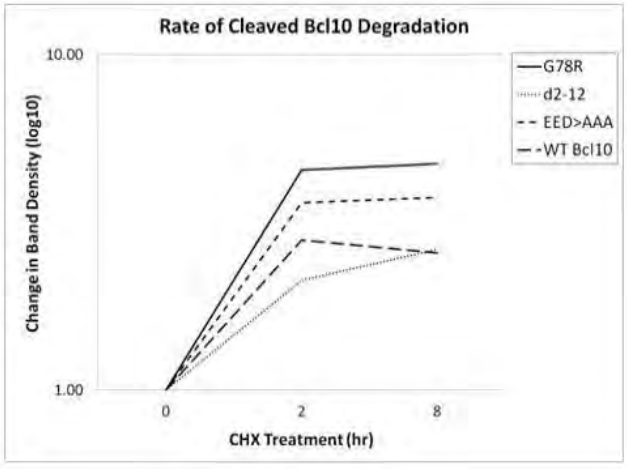

Rate of Cleaved Bcl10 Degradation
Change in Band Density (log10)
10.00
1.00
0
2
8
CHX Treatment (hr)
G78R
d2-12
EED>AAA
WT Bcl10

Figure 6: Mutation of the N-terminal peptide diminishes Bcl10 protein stability. (A) D10 T cells stably transduced with a given Bcl10 construct, were either treated with a DMSO (vehicle control) or 50 μg of cyclohexmide (CHX) for the time points given. Cells were collected as whole cell lysate and run on an SDS-PAGE gel. Gels were transferred to a nitrocellulose membrane for immunoblotting. An anti-Bcl10 antibody was used to probe for total Bcl10 protein, a probe for GAPDH was used as a loading control, and a probe for cyclin D3 as a control for CHX treatment. (B) HEK-293T cells were transiently transfected with a given pcDNA3 3xHA-Bcl10 construct. On Day 2 post-transfection cells were treated with a DMSO control or 50 μg of CHX for the time points given. Whole cell lysates were collected, run on an SDS-PAGE gel, and subjected to immunoblotting. An anti-Bcl10 antibody was used to probe for total Bcl10 protein and a probe for GAPDH was used as a loading control.

Bcl10 degradation is facilitated by the proteasomal and lysosomal pathways (Figures 7A-C)

Previous researchers have established the Bcl10 protein is degraded by complex mechanisms that depend on the state of cellular activation and the modification of Bcl10 by phosphorylation and ubiquitination. A 2007 study by Lobry *et al* [60] supports the conclusion of Bcl10 degradation by the proteasome, while research by Scharschmidt *et al* in 2004 [62] proposed Bcl10 degradation is linked to the lysosomal pathway. Recent work by members of our group [61] has identified a pathway of Bcl10 degradation that is TCR stimulation-dependent and which leads to selective autophagy of Bcl10. It is likely that the major findings of each of these studies is correct, and that Bcl10 degradation is complex and dependent on cell type, cell activation state, and post-translational modification of Bcl10. I wished to determine if the N-terminal peptide of Bcl10 influences the mechanism of Bcl10 degradation. To assess whether the Bcl10 N-terminal peptide has an effect on targeting the protein to either the proteasomal or selective autophagosomal degradation pathways, I used the inhibitors MG132 (an inhibitor of the 26S proteasome), Bafilomycin A1 (an inhibitor of lysosomal acidification and autophagosome-lysosome fusion by blocking H^+ATPase pump function) [118], or E64d (an inhibitor of lysosomal proteases) as a means of blocking protein degradation by the proteasome and autophagosome/lysosome, respectively. Upon treatment of the cells with inhibitor (e.g.

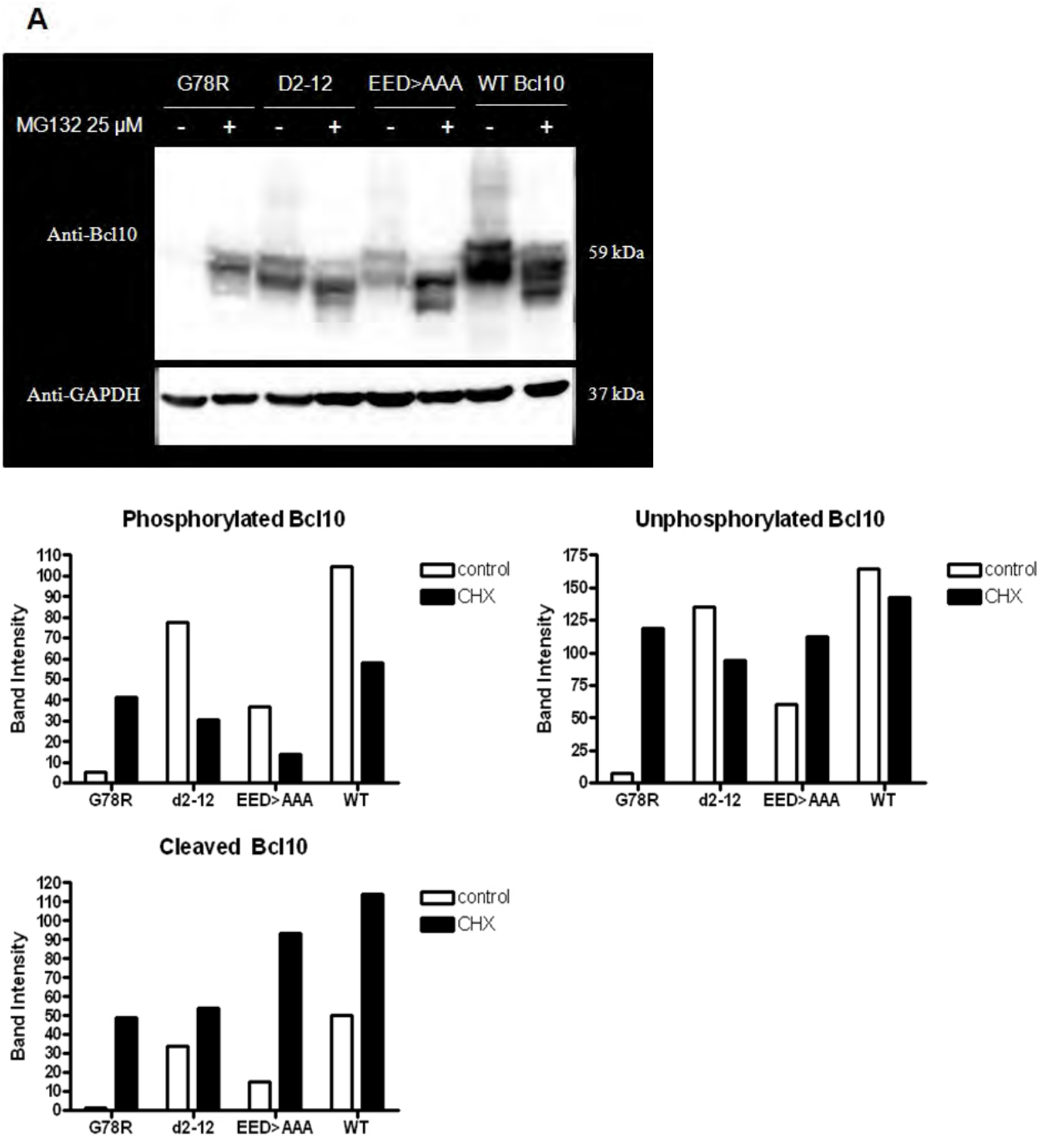
Bafilomycin) I expected that if the Bcl10 protein did not accumulate then the protein was not dependent on that pathway (e.g. autophagosome) for degradation and rather the second pathway (e.g. proteasome) was the protein's primary mode of degradation. As it is likely that Bcl10 follows more than one mechanism of degradation, this assay sought to establish whether the Bcl10 N-terminal peptide was critical to Bcl10 degradation by a particular pathway, and if so, approximately what percentage of the protein follows an N-terminal peptide domain-dependent degradation program.

Stable D10 T cell lines and HEK-293T cells transiently transfected with a given pcDNA3 3xHA Bcl10 construct were both utilized in the following experiments. On the day after transfection, HEK cells were either treated with DMSO (vehicle-only control), with 25 μM of MG132, or with 10 μM E64d. D10 T cells were treated for the times stated with either a DMSO control, 25 μM of MG132, or 50 nM of Bafilomycin. Cells treated with E64d or MG132 were collected as whole cell lysate 16-20 hours after the addition of the inhibitor while Bafilomycin-treated cells were collected as whole cell lysate 5 hours after inhibitor addition. Whole cell lysates were run on an SDS-PAGE gel and subjected to western blotting in a manner described above. Blots were probed with antibodies against Bcl10 and GAPDH in a sequential manner. GAPDH was used as a loading control.

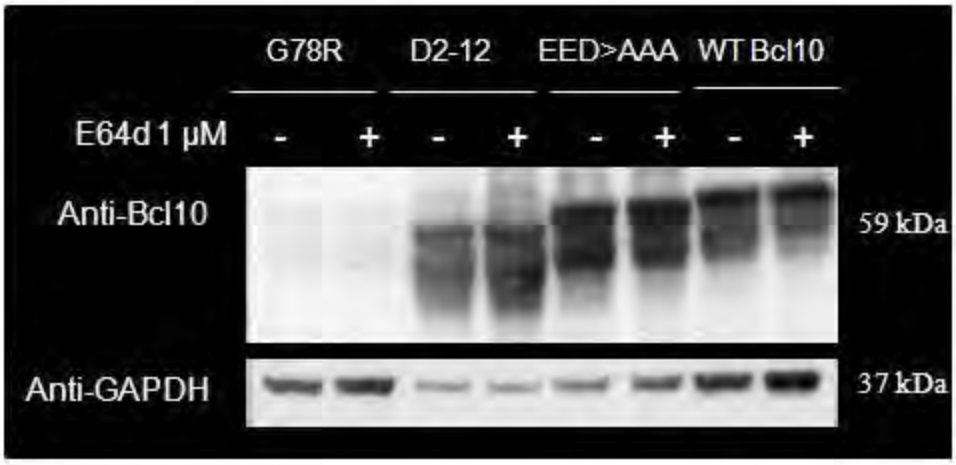
The results of the MG132 (Figure 7A and data not shown), E64d (Figure 7B) and Bafilomycin (Figure 7C) assays yielded results that lend support to previously published reports that Bcl10 degradation involves contributions by the proteasomal and lysosomal pathways. It was observed that Bcl10 protein accumulated upon inhibition of either the proteasomal (Figure 7A and data not shown) or autophagosomal/lysosomal (Figure 7B and 7C) pathways by treatment with MG132 or E64d/Bafilomycin, respectively. Importantly, each inhibitor caused

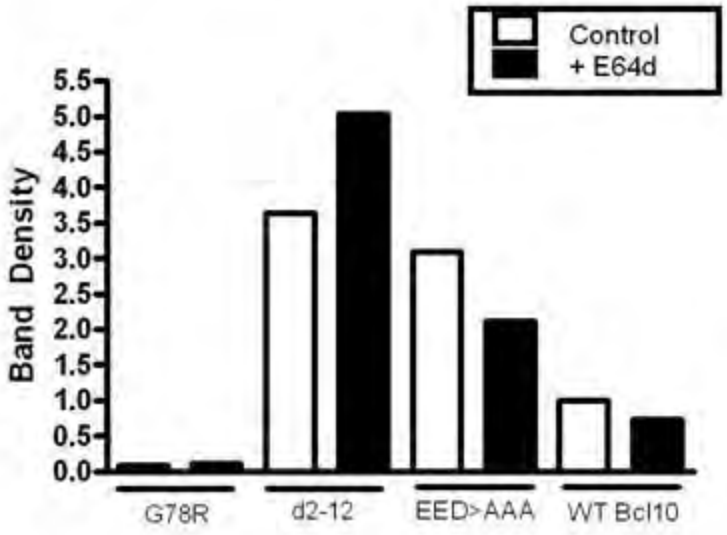
the accumulation of distinctly different Bcl10 protein isoforms. The expectation with MG132 treatment is that a protein that is normally degraded by the proteasome will increase in intensity with MG132 treatment. However, hyperphosphorylated Bcl10 isoforms decrease in intensity upon MG132 treatment (Figure 7A). The decrease in hyperphosphorylated Bcl10 protein concentration after MG132 treatment is in agreement with the results of the CHX assays (Figure 6B), which show that hyperphosphorylated Bcl10 isoforms are rapidly degraded. Accumulation of the cleaved forms with MG132 treatment demonstrates that the cleaved form(s) of Bcl10 are subject to proteasomal degradation (Figure 7A). Notably, all forms of the G78R mutant accumulate dramatically with MG132 treatment, consistent with the expected rapid ubiquitination and proteasomal degradation of this misfolded CARD mutant. These data again emphasize that d2-12 is not a misfolded protein, as it does not exhibit rapid accumulation with MG132 treatment. Still unexplained is the decrease in hyperphosphorylated Bcl10 isoform protein after MG132 treatment. One possible explanation is that the protease responsible for Bcl10 degradation is somehow stabilized by MG132 treatment (e.g. this protease is also a rapidly turned over by the proteasome). Based on the E64d and Bafilomycin inhibitor assays, the Bcl10 isoforms most readily degraded by the autophagosome or lysosome (Figure 7B and 7C) were those that were hyperphosphorylated (following cleavage by an unknown protease); while those degraded by the proteasome (Figure 7A) were different and tended to be of lower molecular weight. Although there are some observable differences in the Bcl10 protein degradation patterns between D10 T cells and HEK cells, it may be possible that different cell types turnover Bcl10 via individual mechanisms depending on the stage of growth and differentiation of the cell. The lower molecular weight Bcl10 bands observed upon MG132 treatment could be due to one or more isoforms of the Bcl10 protein that have undergone proteasome-independent protease

cleavage, possibly by its binding partner MALT1, which is a paracaspase known to cleave Bcl10 following TCR stimulation [37].



B


G78R
D2-12
EED>AAA
WT Bcl10
E64d 1 µM
- + - + - + - +
Anti-Bcl10
59 kDa
Anti-GAPDH
37 kDa


Control
+ E64d
Band Density
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5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
G78R
d2-12
EED>AAA
WT Bcl10

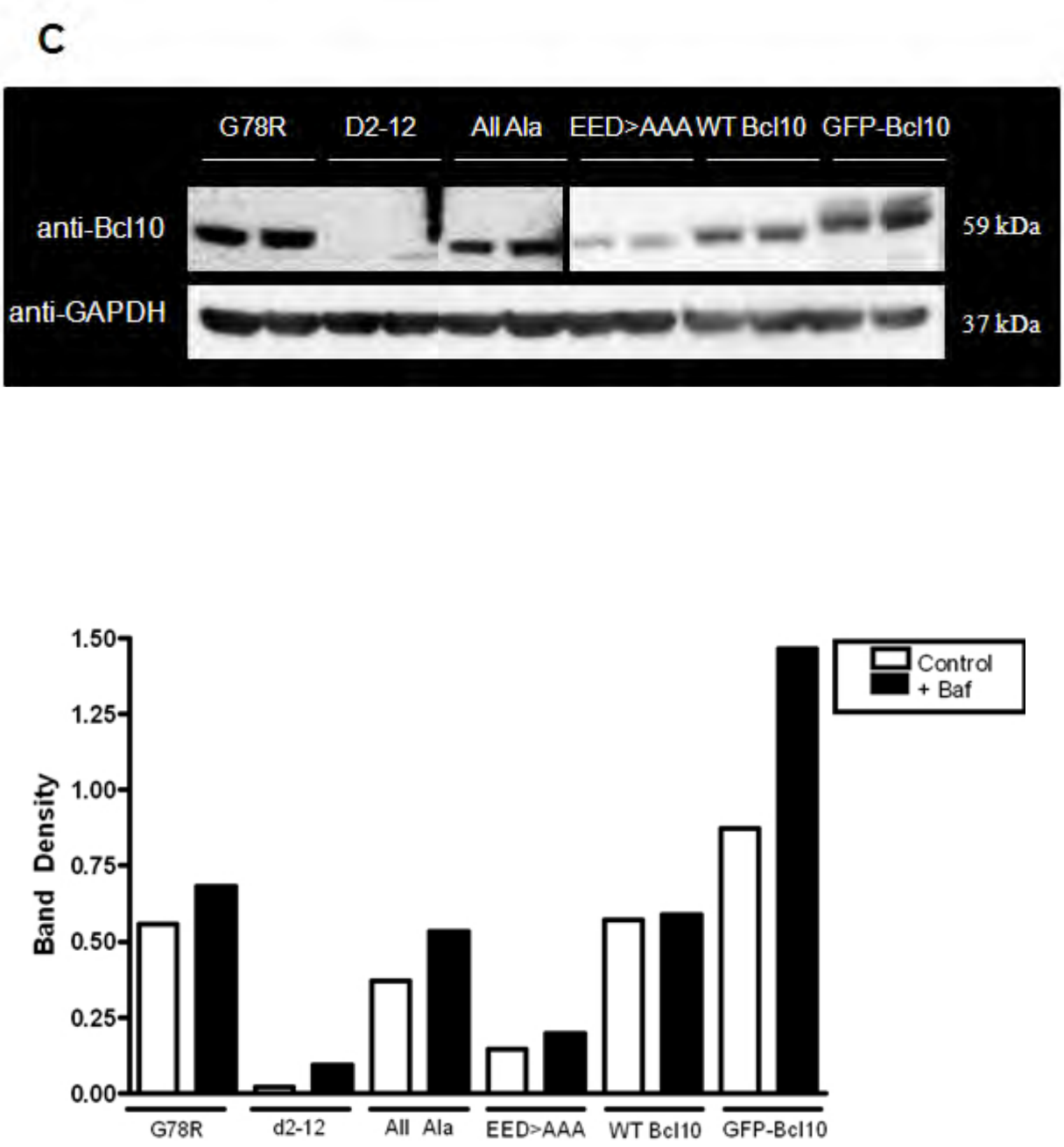


Figure 7: Bcl10 is degraded by both the proteasomal and lysosomal pathways. (A, B) HEK-293T cells were transiently transfected with one pcDNA3 3xHA-Bcl10 construct. On Day 2 after transfection cells were treated with either a DMSO (vehicle-only control), with (A) 25 μM of MG132, or with (B) 10 μM E64d. Cells were collected as whole cell lysate 16-20 hours after the addition of inhibitor. (C) D10 T cells stably transduced with one Bcl10-GFP construct were treated with either a DMSO control or 50 nM of Bafilomycin. Cells were collected as whole cell lysate 5 hours after the addition of inhibitor. (A-C) Whole cell lysates were run on an SDS-PAGE gel and subjected to immunoblotting. Blots were probed with antibodies against Bcl10, and GAPDH as a loading control.

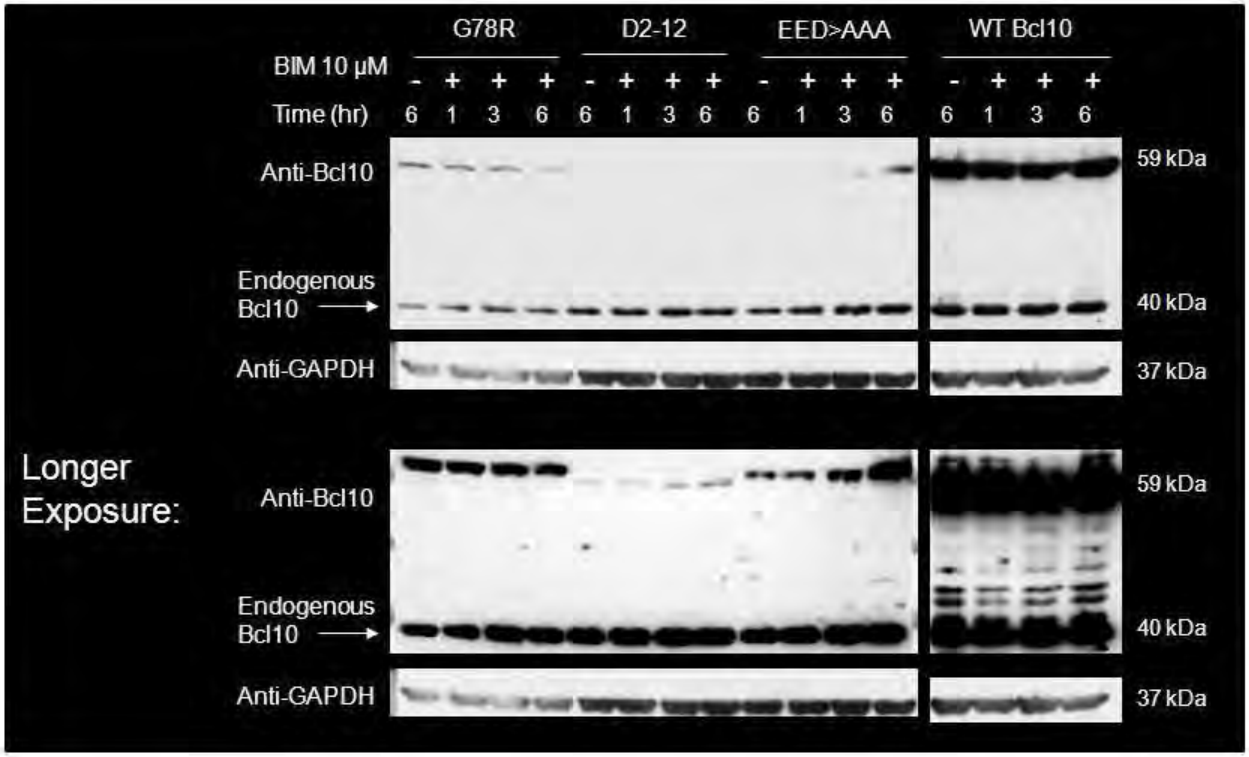
Bcl10 N-terminal peptide mutation destabilizes hyperphosphorylated Bcl10 isoforms (Figures 8A and 8B)

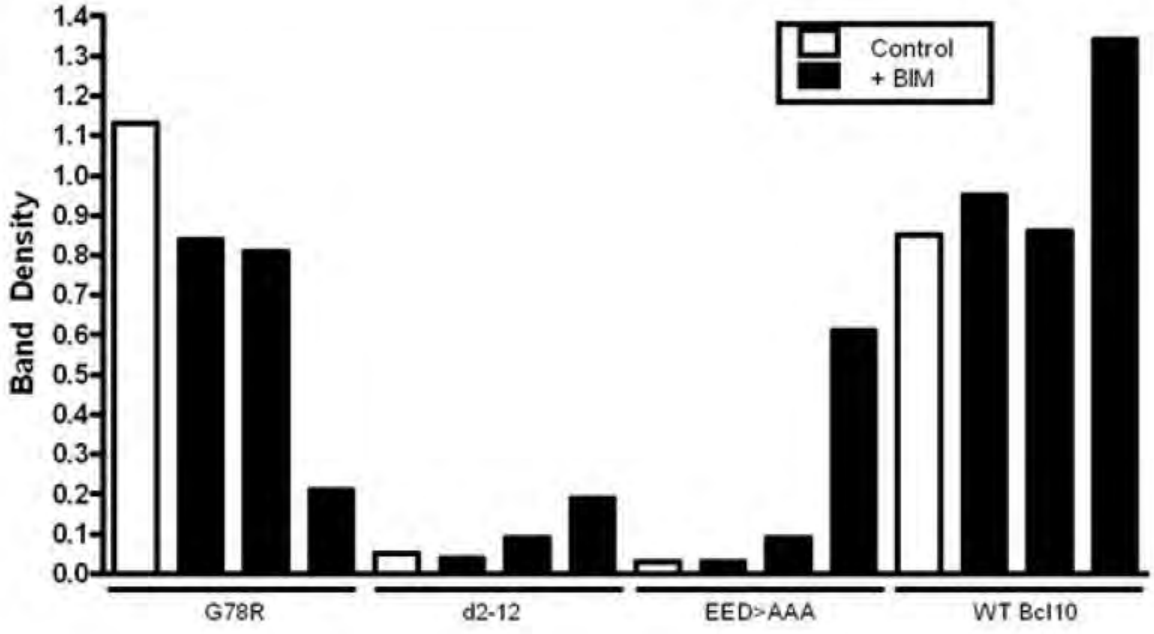
Studies of Bcl10 phosphorylation have highlighted the Bcl10 Serine-Threonine-rich C-terminal domain as being a region enriched in potential phosphorylation sites [55]. The functions of Bcl10 phosphorylation sites are diverse, with apparently conflicting reports of functional importance by different research groups [55]. For example, some groups report sites of Bcl10 phosphorylation as enhancing NF-κB activation, and other groups have suggested Bcl10 phosphorylation is a mechanism of disassociating Bcl10 binding partner proteins (e.g. MALT1) [111], and/or attenuating NF-κB signaling [119]. However, given substantial data that Bcl10 phosphorylation plays important roles in Bcl10 biology, I performed experiments to assess whether the Bcl10 N-terminal influences Bcl10 phosphorylation, and whether Bcl10 phosphorylation influences the rate of Bcl10 degradation. At least one previous study by Lobry *et al* [60] highlighted S7 as a possible Bcl10 N-terminal peptide phosphorylation site. My own site-directed mutagenesis of prospective Bcl10 N-terminal peptide phosphorylation sites indicated that S7 and T9 are not likely to be sites of Bcl10 phosphorylation important for NF-κB activation (Figure 1B). I therefore was interested in determining if the Bcl10 N-terminal peptide influences the prevalence of Bcl10 isoforms that exhibit hyper-phosphorylation upon absence or mutation of the N-terminal peptide (e.g. d2-12 Bcl10). Using the broad spectrum protein kinase C (PKC) inhibitor, bisindolylmaleimide (BIM), I sought to determine if it was possible to block Bcl10 N-terminal peptide mutation-induced hyper-phosphorylation and whether inhibition of Bcl10 phosphorylation by this family of kinases would affect Bcl10 degradation kinetics.

I first determined the effect of BIM on the previously described D10 T cell lines. D10 T cells (5×10^6) expressing one stably transduced Bcl10 N-terminal peptide mutant were treated with 10 μM BIM for the time stated. D10 T cells were collected as whole cell lysate (WCL), which was then run on an SDS-PAGE gel and subjected to western blotting as described above. Blots were probed with an antibody against Bcl10 to quantify whole Bcl10 protein from lysates, as well as an antibody against GAPDH as a loading control.

Using previous western blotting data obtained from the MG132 assays (Figure 7B) as a benchmark I compared band densities of hyper-phosphorylated Bcl10 isoforms to those of lower molecular weight Bcl10 isoforms over a time course of BIM cellular treatment. Previous observations showed that mutation of the N-terminal peptide (e.g. d2-12 Bcl10) resulted in hyper-phosphorylated Bcl10 isoforms that were either less pronounced or absent in WT Bcl10 cells (Figure 7B). The D10 T cell BIM assay showed that compared to untreated D10 T cells, which exhibit low or no visible Bcl10 protein bands for the d2-12 Bcl10 mutant on western blots (Figure 8A), the BIM treated D10 T cells showed an increase in Bcl10 protein concentration over time. Both the d2-12 and EED>AAA Bcl10 N-terminal peptide mutants showed a steady increase in Bcl10 protein concentration over the length of BIM PKC inhibitor treatment. Both controls, G78R Bcl10 and WT Bcl10, showed no pronounced difference in Bcl10 protein concentration over the time course of BIM treatment. Although there was some variability amongst experiments with BIM treatment, the results of this assay (Fig 8A) are consistent with the model suggested by data from the CHX and MG132 treatments.

A


G78R
D2-12
EED>AAA
WT Bcl10
BIM 10 µM
- + + + - + + + - + + + - + + +
Time (hr)
6 1 3 6 6 1 3 6 6 1 3 6 6 1 3 6
Anti-Bcl10
Endogenous Bcl10
59 kDa
40 kDa
Anti-GAPDH
37 kDa
Longer Exposure:
Anti-Bcl10
Endogenous Bcl10
59 kDa
40 kDa
Anti-GAPDH
37 kDa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+ BIM
Band Density
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0.0
G78R
d2-12
EED>AAA
WT Bcl10

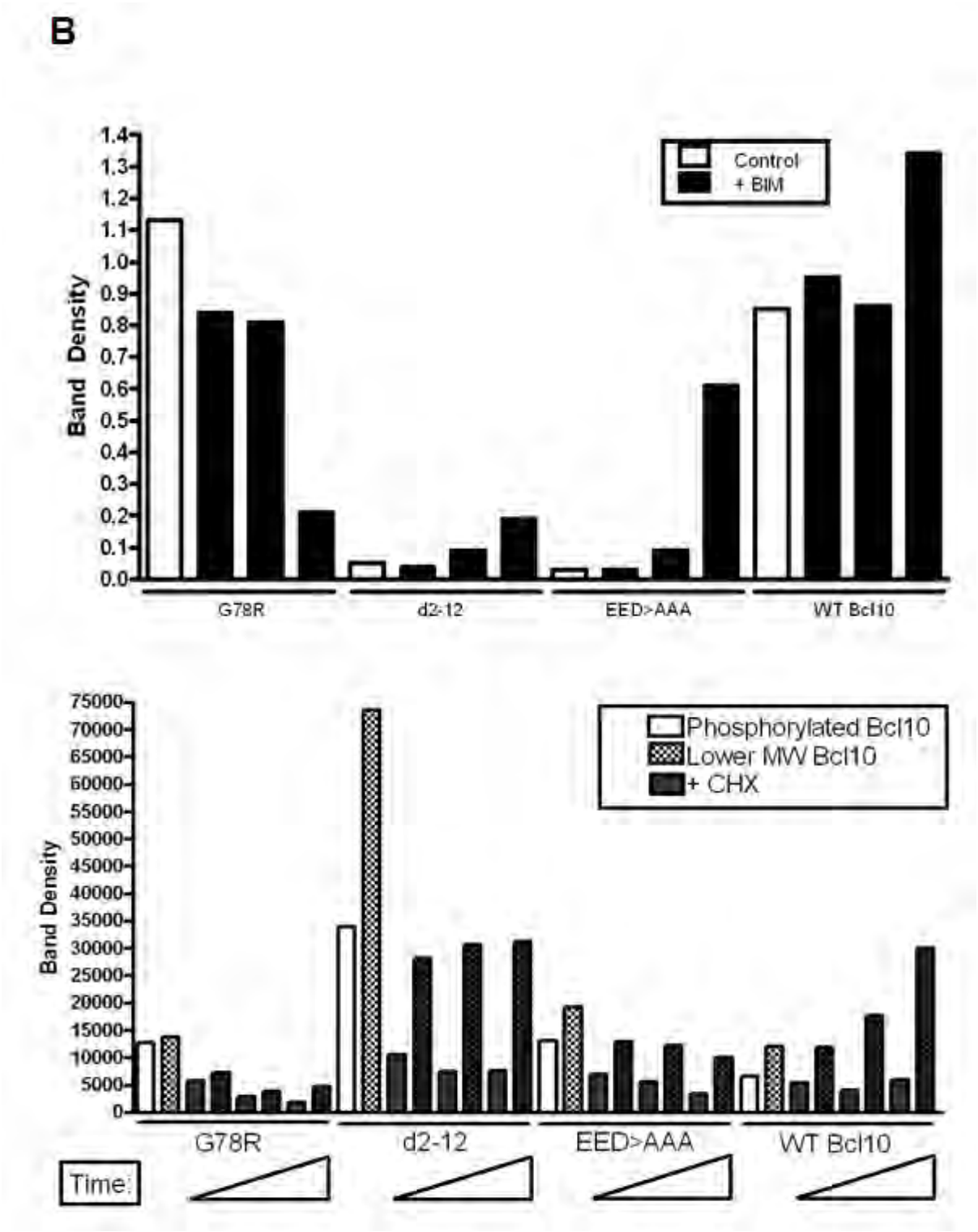


Figure 8: Mutation of the Bcl10 N-terminal peptide affects protein hyperphosphorylation. (A) D10 T cells (5 x 10^6) expressing one stably transduced Bcl10 N-terminal peptide mutant were treated with 10 μM bisindolylmaleimide (BIM) for the time given. Cells were collected as whole cell lysate, which was run on an SDS-PAGE gel and subjected to immunoblotting. Blots were probed with an antibody against Bcl10, and an antibody against GAPDH as a loading control. (B) Upper panel: Graph from Figure 8A; Lower panel: Graph from Figure 6A CHX assay.

The results of the BIM assay suggest that T cells expressing Bcl10 constructs where the N-terminal peptide is either mutated (e.g. EED>AAA Bcl10) or deleted (e.g. d2-12 Bcl10) express hyperphosphorylated Bcl10 isoforms at a rate much higher than WT Bcl10. I speculated that Bcl10 is degraded very rapidly following phosphorylation, and thus the hyperphosphorylated isoforms cannot be detected via western blotting. Upon treatment with BIM, D10 T cells expressing N-terminal peptide mutant Bcl10 constructs exhibited a time-dependent accumulation of Bcl10 protein that was not observed in control cells. When the D10 T cell BIM assay results are compared to the T cell CHX assay results (Figure 8B, lower panel) I noticed an overall trend linking the N-terminal peptide mutants that are most susceptible to degradation after CHX treatment being those most quickly accumulated upon cessation of phosphorylation after BIM treatment. A simple model to explain these data is that one or more PKC kinases either directly or indirectly affect the hyperphosphorylation of Bcl10. Furthermore, one role of the N-terminal peptide is to protect Bcl10 from this hyperphosphorylation in the steady state. This hyperphosphorylation of Bcl10 causes N-terminal peptide mutants to undergo rapid cleavage to lower molecular weight Bcl10 isoforms by a yet unknown mechanism, followed by proteasomal degradation. Upon blockade of Bcl10 hyperphosphorylation via BIM treatment, we are able to visualize the time-dependent accumulation of N-terminal peptide mutants of the Bcl10 protein (Figure 8A). These mutants would otherwise undergo rapid degradation, as seen in the CHX assays (Figure 6A). The BIM assay results overall lead me to believe that the Bcl10 N-terminal peptide is not phosphorylated itself but rather functions to protectively inhibit Bcl10 from undergoing hyper-phosphorylation, which appears to be a state highly susceptible to premature protein degradation and/or cleavage.

Discussion

Developments in NF-κB signaling downstream of the T cell receptor, as well as many other cellular receptors, have been numerous over the past decade [94]. Characterization of NF-κB signal transduction has included both research into the minutia of protein domain interactions, such as those within the CBM complex [36], and also research into the ramifications of unchecked NF-κB signaling [94]. The Bcl10 protein has been highlighted by many researchers for its importance in both the NF-κB signaling cascade in T cells [53] and other cellular processes, including cell motility [56]. The research presented here was carried out to better define the Bcl10 domain dubbed the N-terminal peptide, in the hopes of determining its functional significance to the Bcl10 protein. As there is currently no crystal structure of the Bcl10 protein, it is difficult to extrapolate the protein's important conformational structures, except via hypothetical computer modeling [54]. Bcl10 contains a structural motif called a CARD domain, which is a protein-protein interaction domain found in approximately 40 other proteins [46]. However, the conserved N-terminal peptide of Bcl10 shares no known homologous sequence with any other protein (including other CARD proteins), making it both highly conserved within the Bcl10 protein over multiple species as well as unique to the Bcl10 protein itself. Using potential sites of modification or binding within the N-terminal peptide as a guide, I developed several deletion and missense mutants (Table 1) of this domain to better characterize its functional importance to NF-κB activation, Bcl10 phosphorylation, Bcl10 cellular translocation, and Bcl0 stability and degradation.

Due to the importance of Bcl10 to proper downstream NF-κB activation, an NF-κB inducible luciferase promoter was transfected into HEK-293T cells in conjunction with a reporter

gene and one Bcl10 N-terminal peptide mutant. These experiments allowed for the determination of which N-terminal peptide mutations were deleterious to the function of Bcl10 in activating downstream NF-κB. Using WT Bcl10 as a positive control and the misfolded CARD mutant G78R Bcl10 [112] as a negative control, I assessed over a dozen N-terminal peptide mutants for their ability to activate NF-κB. Only one amino acid change in the N-terminal peptide of Bcl10 was necessary to incur a significant decrease in NF-κB activation, relative to WT Bcl10 (data not shown). This includes the A5S Bcl10 mutant that has been associated with contributing to cell transformation leading to disease [104], but which only showed a nonstatistically significant impairment in ability to activate NF-κB according to NF-κB inducible luciferase promoter assays. Overall, the NF-κB luciferase assays gave a broad understanding of the regions of importance within the Bcl10 N-terminal peptide.

Although the N-terminal peptide is a unique domain, it appears that it cannot bind to or affect downstream NF-κB activation, without the remainder of the Bcl10 protein, as evidenced by the 1-12 only Bcl10 mutant (Figure 1A). Steric hindrance of a WT N-terminal peptide (GFP-Bcl10) resulted in a strong impairment in NF-κB activation, which is attributed to either a conformational change imparted to this region from the GFP tag, and/or interference with the ability of a partner protein to interact with a region proximal to the N-terminus of Bcl10 (Figure 1A). Upon completion of these assays, I am confident in stating the N-terminal peptide does not have a phosphorylation site critical to NF-κB activation. Mutation of the potential phosphorylation sites (e.g. S7 or T9) to alanine or to a phosphorylation-mimicking amino acid showed no strong impairment or increase in NF-κB activation (Figure 1B).

The N-terminal peptide is also upstream of the functionally important CARD domain. The last three amino acids directly upstream of the CARD (EED) appear to have a particularly

important role in influencing the function of the CARD, as mutation of all three amino acids to alanines results in a significant decline in NF-κB activation by Bcl10 (Figure 1A and 1B). Given the polarized nature of approximately one third of the amino acids in the N-terminal peptide, it is likely that they may serve as points of interaction with Bcl10 partner proteins, or with Bcl10 itself. The N-terminal peptide, particularly the three amino acids directly upstream of the CARD domain (10-12; EED) may help CARD binding partners of Bcl10 to stabilize their interactions with Bcl10 by providing an additional charged interaction site.

The analysis of d2-12 and All-Ala Bcl10 mutants demonstrated that the length of the peptide alone is not sufficient to maintaining Bcl10 function, and that whether the whole N-terminal peptide is deleted or mutated, there was no observed downstream NF-κB activation by Bcl10. Thus, one or more subgroups of amino acids within the peptide are likely to serve as a binding site for a partner protein (Figures 1A and 1B). Together, these data show that the Bcl10 N-terminal peptide, a novel domain with no known homologous domains in other proteins, is required for Bcl10-mediated activation of NF-κB.

Intracellular redistribution is a defined and characteristic trait of Bcl10, which is triggered by TCR stimulation [33, 34, 60]. Previous research by members of our lab [33, 34] have defined punctuate structures of Bcl10 enrichment known as POLKADOTS that are important to CBM complex formation and downstream NF-κB activation. I therefore stably transduced mouse D10 T cells with the Bcl10 N-terminal peptide mutants which exhibited the strongest phenotypes in the NF-κB luciferase assays. Stimulating the mouse D10 T cell lines with their specific antigen (conalbumin) presented by mouse CH12 B cells induced POLKADOTS formation by WT Bcl10. As expected, no POLKADOTS were formed by the G78R Bcl10 negative control (Figure 2B). Interestingly, I observed a spectrum of phenotypes in the N-terminal peptide mutants. Although

EED>AAA Bcl10 formed POLKADOTS-like structures upon TCR stimulation, these coalesced puncta were weaker in strength and smaller in size than expected for WT Bcl10 POLKADOTS (Figure 2B). Likewise, WT Bcl10 with an N-terminus GFP-tag exhibited a Bcl10 translocation phenotype unlike WT Bcl10, with Bcl10 being localized strongly to the cellular membrane (Figure 2B), a phenomenon observed by other researchers [117], and likely attributed to the location of the GFP-tag.

The Bcl10 N-terminal peptide mutants that failed to translocate and remained diffusely cytoplasmic were the 1-12 only, d2-12, and All-Ala Bcl10 mutants. Without the Bcl10 CARD and Serine-Threonine C-terminal domain I did not expect 1-12 only Bcl10 to translocate to POLKADOTS, and observations were consistent with this expectation. Moreover, 1-12 only Bcl10 exhibited no discernable cellular redistribution upon T cell stimulation (Figure 2B). Analysis of the d2-12 and All-Ala Bcl10 mutants again emphasized that it is not just the length of the Bcl10 N-terminal peptide that is required for Bcl10 function, but rather some functional property of the amino acids within its sequence. At least some of these amino acids (e.g. EED) appear to be critical to Bcl10 cellular translocation and downstream NF-κB activation. The weaker fluorescent signals given by the d2-12 and All-Ala Bcl10 mutants also suggested that these mutants are quickly degraded within the cell. Reduced steady-state protein levels may also contribute to the reduced downstream NF-κB activation by these mutants.

The overall conclusions drawn from the fluorescence microscopy data were that the Bcl10 N-terminal peptide is required for Bcl10 translocation. N-terminal peptide mutants either cannot translocate efficiently (e.g. EED>AAA Bcl10) or cannot translocate at all (e.g. d2-12 and All-Ala Bcl10). As binding to partner proteins is essential for Bcl10 function and translocation leading to downstream NF-κB activation, I hypothesized the N-terminal peptide is required for

binding to one or more Bcl10 binding partners, which are required for Bcl10 redistribution in response to TCR stimulation. To determine if N-terminal peptide mutants act in a dominant-negative fashion on endogenous Bcl10, it would be beneficial to plan future experiments in which T cells are co-transduced with a WT Bcl10-GFP and a Bcl10 mutant (e.g. d2-12) that had a different fluorescent label (e.g. Red fluorescent protein; RFP). This would allow for determination of whether POLKADOTS formation is inhibited in a dominant-negative manner. In other words, do N-terminal peptide mutants prevent POLKADOTS formation by WT Bcl10, or do these mutants simply fail to form POLKADOTS on their own. Another direction of interest would be to co-transduce Bcl10 with another CBM complex member, such as MALT1 with an RFP tag. Again, this experiment would allow for visualization of POLKADOTS formation by endogenous Bcl10 with MALT1-RFP if N-terminal peptide mutants do not act in a dominant-negative manner.

After observing that GFP fusions of specific Bcl10 N-terminal peptide mutants were poorly fluorescent (Figures 2A and 2B), quantitative analysis of protein concentration was performed. Transduced Bcl10 protein concentration was quantified from whole cell lysates of the D10 T cell lines run on SDS-PAGE gels and western blotted for Bcl10. Relative to WT Bcl10 and many other Bcl10 N-terminal peptide mutants (e.g. EED>AAA, S7AT9A, S7DT9E), the mutants d2-12 and All-Ala Bcl10 showed an approximately 10-fold reduction in transduced Bcl10 protein expression (Figures 3A-C). The results of these protein concentration western blots coupled with the fluorescence microscopy data demonstrated that the d2-12 and All-Ala Bcl10 mutants were expressing transduced Bcl10 protein at a rate substantially lower than transduced WT Bcl10, and that these two mutant proteins are most likely quickly degraded by the cell (since the same expression construct was used for all Bcl10 variants).

Premature degradation of d2-12 and All-Ala Bcl10 could be due to destabilizing the interaction of a binding partner, which normally protects Bcl10 from proteolytic cleavage and/or degradation. Alternatively, the N-terminal peptide may regulate Bcl10 phosphorylation after protein synthesis [57], thereby influencing downstream Bcl10 proteolysis. However, as Bcl10 undergoes numerous modifications via phosphorylation and ubiquitination at sites both known and unknown [55] it is difficult to speculate as to which phosphorylation sites are directly dependent on the N-terminal peptide, and which are most important for regulating Bcl10 stability. Regardless of the precise mechanism, the high turnover rate of the d2-12 and All-Ala Bcl10 mutants likely contributes to a lack of strong downstream NF-κB activation. In contrast, the GFP-Bcl10 construct, which has a WT N-terminal peptide, did not efficiently activate NF-κB in the luciferase assays (Figure 1A), but exhibited higher than WT levels of steady-state Bcl10 protein (Figure 3A). One possibility consistent with the above model is that the N-terminal GFP may sterically hinder the access of a Bcl10-cleaving protease that plays a major role in Bcl10 degradation. Thus, the difference between high turnover Bcl10 variants (e.g. d2-12 and All-Ala Bcl10) and the low turnover GFP-Bcl10 construct may be attributable to changes in the ability of a binding partner (such as a Bcl10-cleaving protease) to functionally associate with Bcl10, thus leading to an alteration in the Bcl10 degradation process.

The Bcl10 protein interacts in a dynamic manner with several binding partners involved in the NF-κB signaling cascade. Bcl10 is ubiquitously associated with the CBM complex member MALT1, which interacts with the Bcl10 fifth and sixth CARD helices as well as a 13 amino acid-stretch directly downstream of the CARD [30, 54]. Bcl10 also interacts with many other CARD proteins including the CARMA proteins (e.g. CARMA1 in hematopoietic cells) [32], and the equine herpes virus protein vE10 [77]. Bcl10 also homodimerizes or homo-

oligomerizes with itself, particularly following TCR stimulation [34]. As no known Bcl10 partner protein binds to the N-terminal peptide, I investigated the functional significance of the N-terminal peptide with regards to binding partners critical to the NF-κB signaling cascade. For this set of experiments I co-transfected one Bcl10 binding partner with one Bcl10 construct in HEK-293T cells and then used co-immunoprecipitation to determine how well each Bcl10 construct could bind to each partner protein. I postulated that the protein most likely to bind well to Bcl10 regardless of the N-terminal peptide sequence (due to its critical binding region being located furthest from the Bcl10 N-terminus) was MALT1. Surprisingly, results showed a propensity for MALT1 to bind Bcl10, dependent on the length of the N-terminal peptide, with longer deletions of the peptide resulting in less MALT1 protein binding (Figure 4B). Despite the 13-amino acid MALT1 binding site being far downstream in the Bcl10 protein sequence, even the EED>AAA Bcl10 construct showed impaired binding to MALT1. It is unlikely that MALT1 interacts with the N-terminal peptide directly, as the GFP-Bcl10 construct did not impair MALT1 co-immunoprecipitation (Figure 4B). I propose that the N-terminal peptide stabilizes MALT1 binding to Bcl10 via an indirect mechanism, possibly by controlling the post-translational modification of residues in the Malt1 binding site (e.g., helix 5-6). I next analyzed the Bcl10 CARD binding partners CARMA1, CARMA3, vE10, and WT Bcl10 (Figures 4A, 4C, 4D and 4E). With the exception of CARMA3, all of the CARD partner proteins exhibited impaired binding to the N-terminal peptide mutants d2-12 and EED>AAA Bcl10. CARMA1 showed particularly weak binding to d2-12 Bcl10, whereas CARMA3 binding to d2-12 Bcl10 showed little to no impairment (Figures 4A and 4E). The difference in binding aptness between the paralogous CARMA proteins proves that the N-terminal peptide of Bcl10 functions somewhat uniquely in different cell types. CARMA1 functions strictly in the NF-κB pathway

downstream of the TCR or B-cell receptor of hematopoietic cells [23, 29], while CARMA3 functions in the NF-κB pathway downstream of numerous signaling receptors of non-hematopoietic cells [48, 49, 120]. WT Bcl10 and the protein vE10 also could not bind EED>AAA Bcl10 at a level comparable to WT Bcl10-GFP (Figures 4C and 4D). These results suggest the N-terminal peptide strongly influences the function of the Bcl10 protein, via controlling interactions with a large number of partner proteins.

The conclusions drawn from the co-immunoprecipitation data led me to believe that some CARD proteins rely more heavily on the function of the N-terminal peptide to bind Bcl10 efficiently than other CARD proteins (e.g. CARMA1 *vs.* vE10 or CARMA3). Given that vE10 is known to strongly associate with Bcl10 and translocate cellular Bcl10 to the cell membrane [80] I revisited the question of the function the N-terminal peptide has in vE10-mediated Bcl10 translocation. Previous research has shown that vE10 acts dominantly on Bcl10 translocation, redistributing the protein pair to the cell membrane [80]. I co-transduced the D10 T cell lines with vE10 and either left the cells unstimulated or stimulated them with their specific antigen. It was observed that the Bcl10 N-terminal peptide mutants that exhibited an inability to form POLKADOTS in stimulated T cells (Figure 2B) could readily co-localize with vE10 (Figure 5B). Indeed, Bcl10 co-localization with vE10 superseded POLKADOTS formation in stimulated T cells, highlighting the ability of a CARD binding partner to interact with Bcl10 N-terminal peptide mutants with deleted (e.g. d2-12 Bcl10) or severely mutated (e.g. All-Ala Bcl10) N-terminal peptides. The conclusions I can draw from this set of experiments is that the Bcl10 N-terminal peptide is not required for efficient binding of at least one Bcl10 partner protein (e.g. vE10). Although the co-immunoprecipitation data (Figure 4D) indicated that the Bcl10 N-terminal peptide augments binding to vE10, the fluorescence microscopy data demonstrate that

the N-terminal peptide is not an absolute requirement for binding to vE10. Overall, I believe that the N-terminal peptide influences the conformation and/or the post-translational modification of Bcl10, with a strong effect on binding of some partners (e.g., CARMA1 and Malt1) and a small effect on other partners (e.g., CARMA3, vE10).

As previously discussed, some Bcl10 N-terminal peptide mutants exhibited a lower cellular protein concentration that I had attributed to either degradation or protein cleavage (Figure 3A-C). Using cycloheximide (CHX), an inhibitor of protein translation, I was able to determine the degradation rates of Bcl10 N-terminal peptide mutants during steady-state growth. In the D10 T cells it was noted that the d2-12 and All-Ala Bcl10 mutants exhibited degradation rates far higher than those for WT Bcl10 and GFP-Bcl10 (Figure 6A). The HEK CHX assays (Figure 6B) however, helped to define the subgroups of Bcl10 isoforms as being either phosphorylated, un-/under-phosphorylated, or lower molecular weight (e.g. degraded). These three subgroups of Bcl10 isoforms exhibited distinct patterns for each Bcl10 construct. However, of particular prominence was the degree with which phosphorylated isoforms of the N-terminal peptide mutants (e.g. d2-12 and EED>AAA Bcl10) were degraded while at the same time degraded isoforms for these constructs readily accumulated within the cells (Figure 6B). From these assays I first concluded that Bcl10 N-terminal peptide mutants readily undergo post-translational modification, including the previously defined hyperphosphorylation. Second, I concluded that phosphorylated Bcl10 is targeted for rapid cleavage by a protease. When the N-terminal peptide is mutated, the phosphorylated Bcl10 isoforms may be formed more readily or cleaved more readily by one or more proteases, resulting in degradation, as seen in Figure 6B. Lastly, data from CHX assays in conjunction with those of the protein expression assays (Figure 3) demonstrated that mutation (e.g. EED>AAA) or steric hindrance (GFP-Bcl10) of the N-

terminal peptide do not necessarily impair protein expression or half-life. Both EED>AAA Bcl10 and GFP-Bcl10 showed protein expression comparable to WT Bcl10, if not higher (Figure 3A). However, both of these mutants showed a significantly impaired ability to activate NF-κB, as seen in the luciferase assays (Figure 1). Taken together these data strongly suggest that good Bcl10 protein expression does not guarantee strong NF-κB activation, but rather NF-κB activation is dependent on a WT N-terminal peptide.

As the mode of Bcl10 degradation has been researched previously with several different conclusions, including proteasomal [60], lysosomal [62], and autophagosomal mechanisms [61], I sought to determine which mechanism makes the greatest contribution to the degradation of the key N-terminal peptide mutants (e.g. d2-12 Bcl10, All-Ala Bcl10, and EED>AAA Bcl10). Using the proteasomal inhibitor MG132, and the lysosomal degradation inhibitors E64d and Bafilomycin, I was able to discriminate between proteasomal and lysosomal degradation mechanisms. Although I observed some divergences in protein degradation dependent on cell type (e.g. D10 T cells *vs.* HEK-293T cells), an overall mechanism of degradation profile is strongly suggested by the results obtained from Figures 7A-C. As seen with the CHX assays, Bcl10 proteins appear to form three distinct subgroups of phosphorylated, un-/under-phosphorylated, and lower molecular weight (e.g. degraded) isoforms. These experiments demonstrated that the lowest molecular weight (cleaved) Bcl10 proteins accumulated after treatment with the proteasomal inhibitor MG132, whereas lysosomal inhibitors had only a small effect, causing slight accumulation of higher molecular weight (unphosphorylated and phosphorylated) forms (Figures 7B and 7C). Given these results, I hypothesized that the lower molecular weight isoforms of Bcl10 are the cleaved products of the unstable higher molecular weight (phosphorylated) Bcl10 proteins, which are shuttled to the proteasomal pathway after

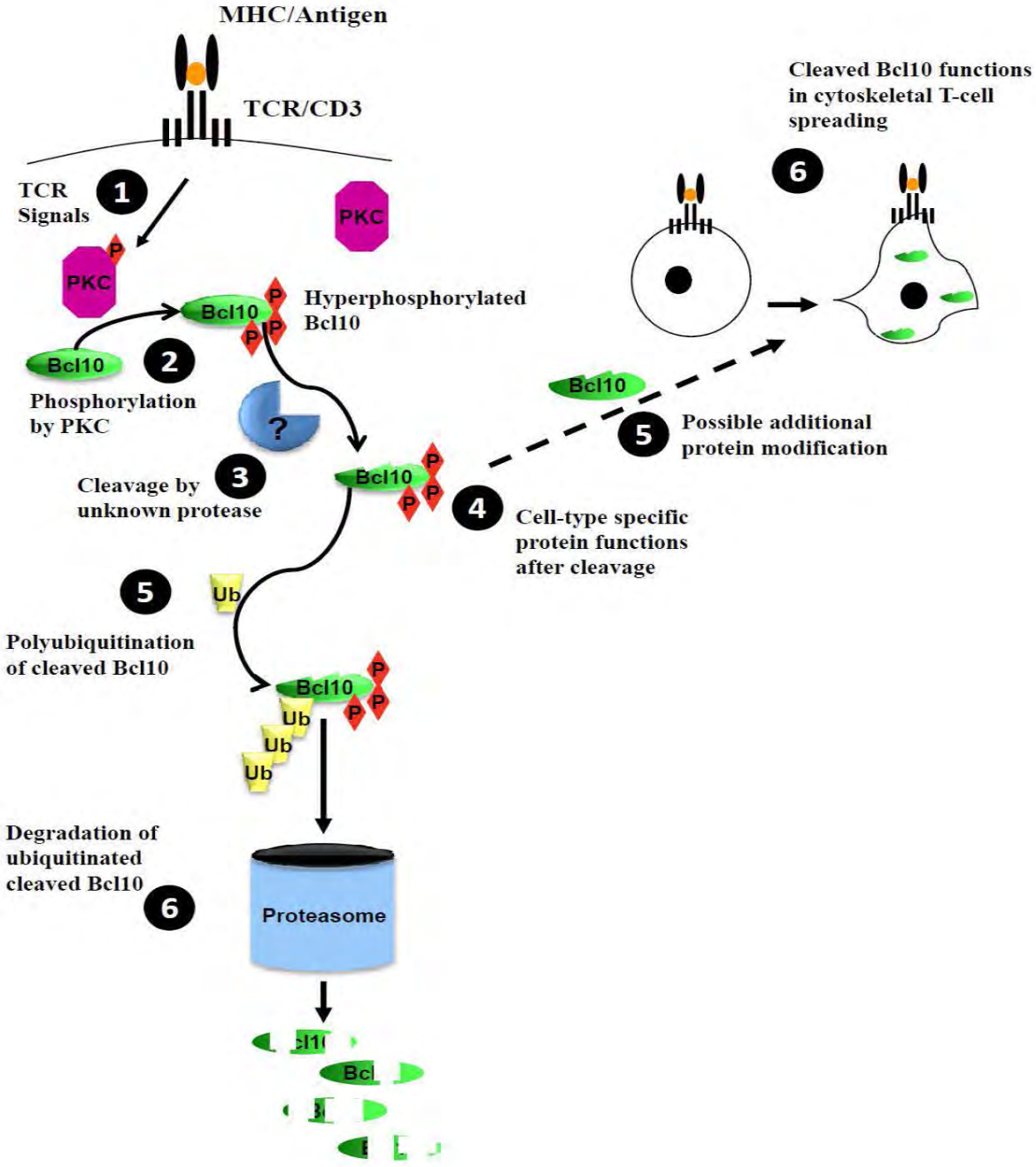
cleavage and ubiquitination (Figure 7A). MG132 western blots were probed for an anti-polyubiquitin antibody to observe if there was an accumulation of ubiquitin-tagged Bcl10 when cells were treated with MG132. However, these blots yielded inconclusive results, most likely due to the challenging nature of this protocol. Because the phosphorylated isoforms of Bcl10 are actually *decreased* by MG132 treatment, the phosphorylated forms are unlikely to be directly degraded by the proteasome. As Bcl10 is known to undergo regulated cleavage by at least one binding partner (e.g. MALT1) [37], there is strong precedence for the hypothesis that Bcl10 is cleaved in a proteasome-independent manner.

Previous data suggested that higher molecular weight isoforms of Bcl10 seen in the CHX (Figure 6B) and MG132 assays (Figure7A) are phosphorylated forms of Bcl10 [33], modified by one or more members of the protein kinase C (PKC) family of kinases. I thus performed experiments using bisindolylmaleimide (BIM), a PKC inhibitor. BIM treatment of D10 T cells gave strikingly different results between WT Bcl10 and N-terminal peptide mutant constructs (Figure 8A). These data suggest that one or more PKC kinases either directly or indirectly control the hyperphosphorylation and concomitant degradation of Bcl10 associated with N-terminal peptide mutation. Over a time course of BIM treatment, the usually weakly expressed d2-12 and EED>AAA Bcl10 proteins increased in concentration while the G78R and WT Bcl10 proteins showed little to no difference in protein expression (Figure 8A). These results corresponded very well (Figure 8B) with those of the CHX assays (Figure 6) showing the quicker degradation of high molecular weight Bcl10 isoforms. Given previous research [33], the conclusion made here, that mobility shifts of Bcl10 isoforms in the SDS-PAGE gels and western blots (Figures 6 and 8) to higher molecular weights are due to Bcl10 phosphorylation, is the most logical explanation. This conclusion is only presumptive however, as these bands were not

directly shown to be phosphorylated. The hyperphosphorylation of Bcl10 by PKC is one mechanism that could explain the absence of hyperphosphorylated Bcl10 isoforms when cells are treated with BIM. However, other contributions to Bcl10 hyperphosphorylation are likely. To definitively determine the locations and kinase(s) responsible for Bcl10 hyerphosphorylation associated with Bcl10 degradation, it would be informative to conduct a mass spectrometric analysis of Bcl10 in conjunction with mutating prospective phosphorylation sites in Bcl10 constructs that are either WT or contain a mutated N-terminal peptide. Directly identifying phosphorylation sites in conjunction with the use of a broad range of kinase inhibitors to determine the source of phosphorylation, will allow researchers to identify which Bcl10 phosphorylation sites are regulated by the N-terminal peptide.

I propose a model (Figure 9 A-C) in which the N-terminal peptide prevents spontaneous hyperphosphorylation of Bcl10, and that hyperphosphorylated isoforms are unstable, being rapidly cleaved by proteases, followed by ubiquitination and proteasomal degradation (Figure 7A). Via this model (Figure 9A) it is likely that upon activation of the NF-κB signal cascade one or more PKC enzymes phosphorylate Bcl10, enhancing its cleavage by a binding partner for the purpose of degradation (an expected outcome of the NF-κB negative feedback loop) and/or re-purposing of the cleaved Bcl10 isoform in another cellular process (e.g. T cell spreading) [37].

A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TCR/CD3
TCR Signals
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PKC
P
PKC
Bcl10
P
P
Hyperphosphorylated Bcl10
Bcl10
2
Phosphorylation by PKC
?
3
Cleavage by unknown protease
Bcl10
P
P
4
Cell-type specific protein functions after cleavage
Cleaved Bcl10 functions in cytoskeletal T-cell spreading
6
Bcl10
5
Possible additional protein modification
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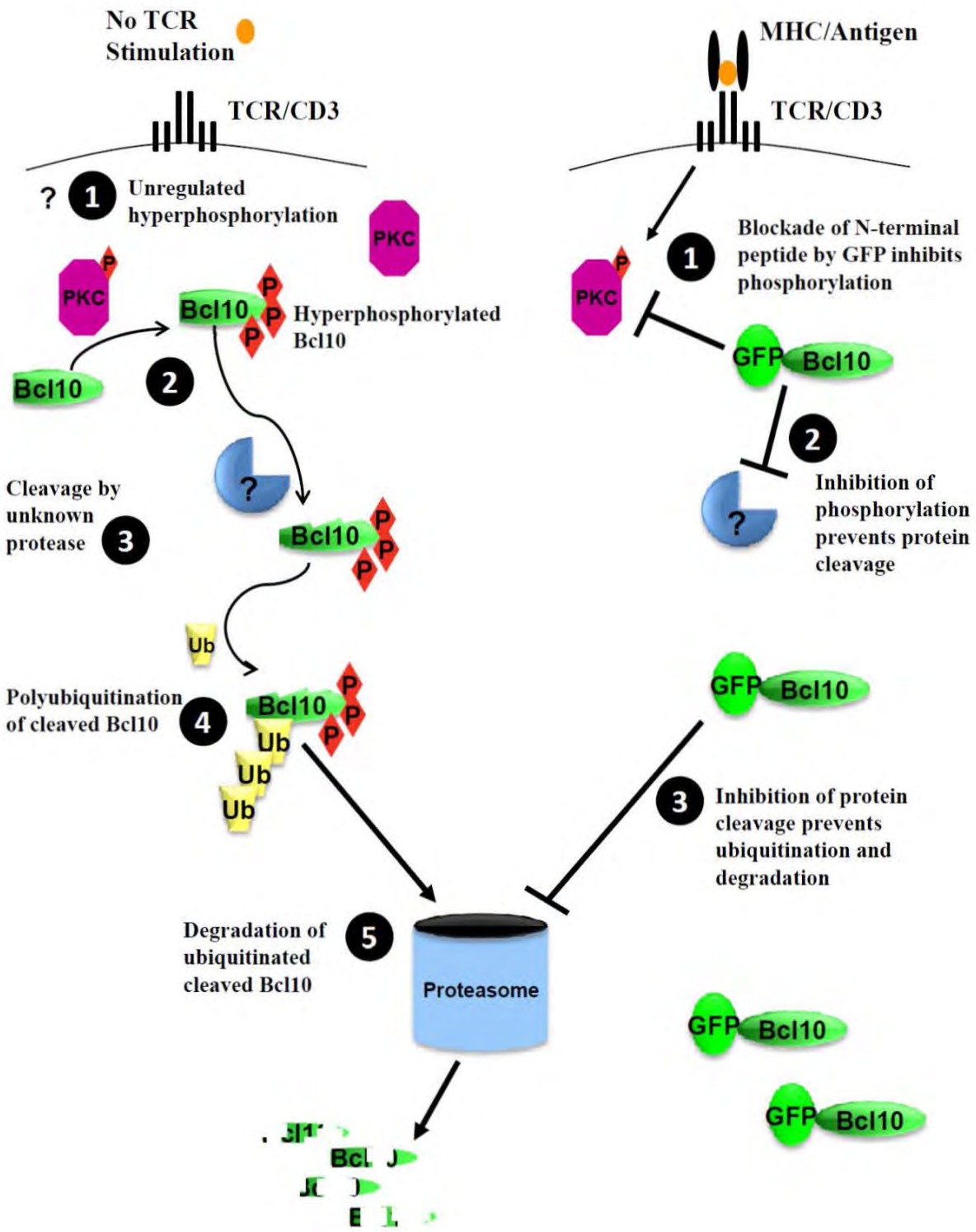
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TCR/CD3
MHC/Antigen
TCR/CD3
1 Unregulated hyperphosphorylation
PKC
Bcl10
Hyperphosphorylated Bcl10
2
Cleavage by unknown protease 3
Polyubiquitination of cleaved Bcl10 4
Ub
1 Blockade of N-terminal peptide by GFP inhibits phosphorylation
GFP Bcl10
2 Inhibition of phosphorylation prevents protein cleavage
3 Inhibition of protein cleavage prevents ubiquitination and degradation
5 Degradation of ubiquitinated cleaved Bcl10
Proteasome

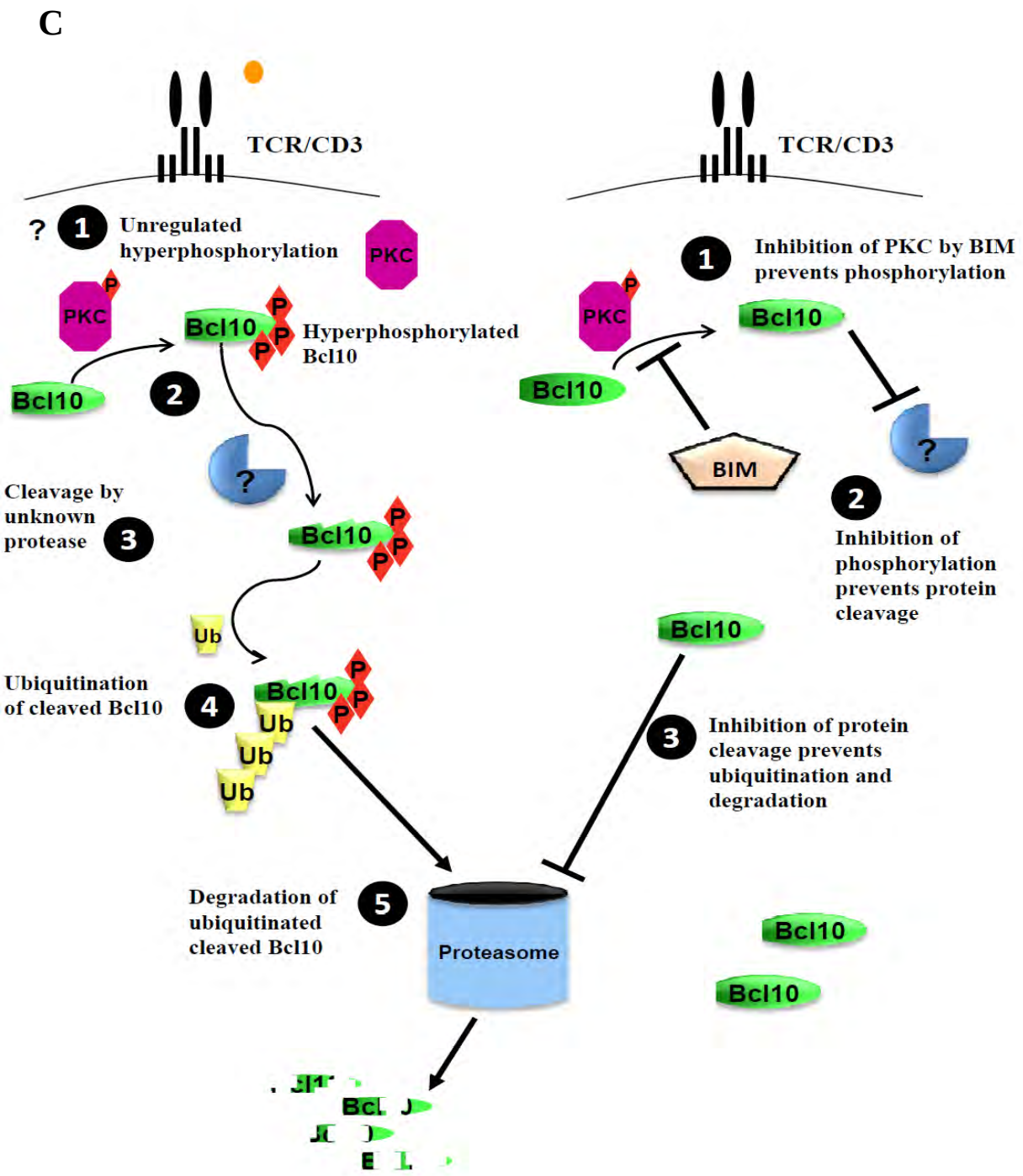

Figure 9. Model of the function of the N-terminal peptide on Bcl10 phosphorylation, cleavage, and degradation. (A) One possible pathway of Bcl10 degradation that results from the NF-κB negative feedback loop downstream of TCR stimulation. (B) (Left) Without a WT N-terminal peptide, Bcl10 is susceptible to unregulated hyperphosphorylation by PKC causing premature degradation and cleavage. (Right) When the N-terminal peptide is sterically hindered (GFP), PKC cannot reach the Bcl10 phosphorylation site, resulting in Bcl10 becoming extremely stable in the steady-state. (C) (Left) Without a WT N-terminal peptide, Bcl10 is susceptible to unregulated hyperphosphorylation by PKC causing premature degradation and cleavage. (Right) Bisindolylmaleimide (BIM) is a PKC inhibitor that prevents Bcl10 hyperphosphorylation in the absence of a WT N-terminal peptide. Inhibition of mutant N-terminal peptide Bcl10 hyperphosphorylation causes steady-state protein stabilization.

I hypothesize the function the N-terminal peptide has in protecting Bcl10 from hyperphosphorylation is to stabilize cytoplasmic Bcl10 in the steady-state. In the absence of a WT N-terminal peptide, Bcl10 is spontaneously phosphorylated, becoming highly susceptible to degradation and/or cleavage (Figure 9B and 9C). The source of this proteolytic cleavage is unknown however, formation of cleaved Bcl10 isoforms was not inhibited upon MG132 treatment, strongly suggesting this protease is not associated with the proteasome. Several Bcl10 binding partners, including MALT1, have been identified as a source of Bcl10 cleavage. It would be beneficial then to perform protein knockdowns or knockouts (e.g. MALT1) to observe if cleaved Bcl10 isoforms are inhibited (then also possibly causing an accumulation of the hyperphosphorylated isoforms) when the N-terminal peptide is mutated.

Both Bcl10 hyperphosphorylation and changes to the primary sequence of the N-terminal peptide may contribute to poor binding to CBM complex members CARMA1 and MALT1, and the resultant poor downstream activation of NF-κB. A definitive Bcl10 crystal structure would be most illustrative to the understanding of how the N-terminal peptide interacts conformationally with the CARD and Serine-Threonine C-terminus domain. The conformation of Bcl10 and the location of the N-terminal peptide, possibly with relation to one or more phosphorylation sites, may address the issues associated with CARMA1 and MALT1 binding being impaired upon mutation of the N-terminal peptide. Analysis of vE10 demonstrated that the N-terminal peptide sequence is not required for all forms of Bcl10 cellular translocation. The results of the vE10 experiments compared with those of CBM complex members show the Bcl10 N-terminal peptide has a specific role in Bcl10 biology, with strong effects on some binding partners and functions, and little to no effect on other partners and activities. The N-terminal peptide is a small domain, and this data suggest that it undergoes no direct modification.

However, the N-terminal peptide may serve as a binding site for one or more proteins as well as protecting Bcl10 from spontaneous hyperphosphorylation in the steady state. Overall, I believe the Bcl10 N-terminal peptide is a worthwhile target of further research, especially as its novel sequence makes it a good candidate for the selective targeting of specific Bcl10 functions and thus NF-κB activation in diseases attributed to NF-κB dysregulation.

Experimental Procedures

Cell lines and reagents. D10 T cells from [121] and CH12 B cells from [122] were maintained as previously described [33, 34]. HEK293T cells were maintained as previously described [54].

Pre-treatments with inhibitors were as follows: Cycloheximide (CHX; Cayman Chemicals) in 95% EtOH (50 μg) for the time points given; E64d (Cayman Chemicals) in DMSO (10 μg/ml), 16 hr; Bafilomycin A1 (Baf; Alexis Biochemicals) in DMSO (25or 50 nM) 5 hr; MG132 (Cayman Chemicals) in DMSO (25 μM), 16-20 hr; Bisindolylmaleimide (BIM; Calbiochem) in DMSO (4 μM or 10μM, as stated) for the time points given.

Cloning and retroviral infections. Gene fusions were cloned into the retroviral vectors MSCV (Clontech; Dr. Robert G. Hawley), pcDNA3 (Invitrogen), pEzeo and pEhyg [113]. Retroviral infection and cell line production were as described [123]. Retroviral transduction and selection of D10 T cells were as previously described [33].

The CARMA1 cDNA was a gift from J. Pomerantz (Johns Hopkins University) and D. Baltimore (Caltech). Deletion and point mutants of Bcl10-GFP fusion proteins were produced via PCR mutagenesis, using standard methodology (Table 1). The Bcl10-dMALT1-GFP was constructed from Bcl10-GFP by deleting residues encoding amino acids 107–119, as previously reported [30]; the G78R Bcl10 mutant was constructed as previously reported [112]; 1-12 only Bcl10 was made by deleting residues encoding amino acids 13-233; d2-4 Bcl10 was made by deleting residues 2-4; d2-9 Bcl10 was made by deleting residues 2-9; d2-12 Bcl10 was made by deleting residues 2-12; EED>AAA Bcl10 was made by PCR mutagenesis of residues 10-12; All Ala Bcl10 was made by PCR mutangesis of residues 2-12 to eleven alanines. MALT1,

CARMA1, Bcl10 and vE10 constructs were fused with the sequence encoding the 3xFLAG epitope, replacing the starting methionine of each with the Met-FLAG epitope tag. Bcl10 constructs were fused with the sequence encoding three repeats of the hemagglutinin (HA) epitope, replacing the starting methionine with the Met-HA epitope tag.

Table 1. Site-directed PCR mutagenesis primers for Bcl10.

Abbr.	Length (bp)	MW	Tm	Name	Sequence
0183	*31*	*9456*	*60*	Bcl10-d8-Anti:Mlu	GGAACGCGTCCCAGTTTTATATTCCGAAGCT
0572	*32*	*9829*	*68*	Bcl10d2-4-Se:Nco	GGACCATGGCACCGTCCCTCACGGAGGAGGAT
0573	*37*	*11555*	*64*	Bcl10d2-9-Se:Nco	GGACCATGGCTGAGGAGGATTTGACTGAAGTGAAGAA
0597	*58*	*17815*	*74*	Bcl10 EED>AAA:NcoI	GGACCATGGAGGCTCCCGCACCGTCCCTCACGGCAG CAGCATTGAC TGAAGTGAAGAA
0598	*31*	*9652*	*60*	Bcl10d2-12:NcoI	GGACCATGGCATTGACTGAAGTGAAGAAGGA
0725	*41*	*12551*	*73*	Bcl10-hu-Nterm mut	GGACCATGGAGCCCACCGCACCGTCCCTCACGGAGG AGGAT
0726	*50*	*15352*	*75*	Bcl10 S7A T9A(mouse)	GGACCATGGAGGCTCCCGCACCGGCACTCGCAGAGG AGGACCTCA CTGAA
0727	*50*	*15391*	*73*	Bcl10 S7D T9E(mouse)	GGACCATGGAGGCTCCCGCACCGGATCTCGAAGAGG AGGACCTCA CTGAA
0728	*47*	*14359*	*75*	Bcl10 S7A(mouse)	GGACCATGGAGGCTCCCGCACCGGCACTCACCGAGG AGGACCTCA CT
0729	*47*	*14374*	*74*	Bcl10 S7D (mouse)	GGACCATGGAGGCTCCCGCACCGGATCTCACCGAGG AGGACCTCA CT
0730	*50*	*15303*	*75*	Bcl10 T9A (mouse)	GGACCATGGAGGCTCCCGCACCGTCCCTCGCAGAGG AGGACCTCA CTGAA
0731	*50*	*15327*	*74*	Bcl10 T9E	GGACCATGGAGGCTCCCGCACCGTCCCTCGAAGAGG AGGACCTCA CTGAA
0732	*47*	*14374*	*74*	Bcl10 P4A	GGACCATGGAGGCTGCAGCACCGTCCCTCACCGAGG AGGACCTCA CT
0733	*47*	*14358*	*73*	Bcl10 P4T	GGACCATGGAGGCTACAGCACCGTCCCTCACCGAGG AGGACCTCA CT
0764	*57*	*17439*	*77*	Bcl10 S7A(BglII)	CCGAGATCTCTCGACGCCACCATGGAGGCTCCCGCA CCGGCACTCA CGGAGGAGGAT
0765	*57*	*17454*	*76*	Bcl10 S7D(BglII)	CCGAGATCTCTCGACGCCACCATGGAGGCTCCCGCA CCGGATCTCA CGGAGGAGGAT
0766	*50*	*15189*	*74*	Bcl10 P4T(BglII)	CCGAGATCTCTCGACGCCACCATGGAGGCTACTGCA CCGTCCCTCA CGGA
0767	*57*	*17365*	*76*	Bcl10 A5S(BglII)	CCGAGATCTCTCGACGCCACCATGGAGGCTCCCTCA CCGTCCCTCA CGGAGGAGGAT
0768	*74*	*22694*	*77*	Bcl10 all Ala(BglII)	CCGAGATCTCTCGACGCCACCATGGCAGCTGCAGCC GCTGCAGCAG

					CTGCTGCCGCATTGACTGAAGTGAAGAA
0769	*68*	*20863*	*78*	Bcl10 1-12(Se)	TCGAGAGATCTCTCGACGCCACCATGGAGGCTCCCG CACCGTCCCTCACGGAGGAGGATGCTGCAGGA
0770	*68*	*20908*	*79*	Bcl10 1-12(anti)	CGCGTCCTGCAGCATCCTCCTCCGTGAGGGACGGTGC GGGAGCCTC CATGGTGGCGTCGAGAGATCTC

Transient transfections were performed by a modified calcium phosphate method [114]. Luciferase assays were done as described [54] and NF-κB activation was assessed as previously described by Lucas *et al.* [30]. Luciferase assays were performed with 112.5 ng of Bcl10-GFP mutant plasmid (cloned into the MSCV vector), 4.5 ng of the NF-κB-responsive luciferase reporter pBVI-NF-κB-Luc (a minimal promoter with multiple NF-κB binding sites; gift from Gabriel Nunez), 49.5 ng of the β-galactosidase reporter pEF1-Bos-βgal (a transfection efficiency control; gift from Gabriel Nunez), 45 ng of pcDNA-p35 (a Bcl-2 homology from baculovirus to promote cell survival), and 388.5 ng of the carrier plasmid, pBS, for a total of 600 ng. Luciferase assays were performed using the Luciferase Assay System (Promega) at 48 hr post-transfection, according to the instructions of the manufacturer. β-Galactosidase assays were performed using the β-Galactosidase Reporter Gene Activity Detection Kit (Sigma). Luciferase values for each sample were normalized to the corresponding β-galactosidase values and reported as NF-κB fold induction relative to control cells transfected with reporter only.

Microscopy. D10 T cells stably expressing Bcl10-GFP constructs with or without FLAG-vE10 were conjugated with conalbumin-loaded CH12 B cells for 20 min at 37 °C, fixed, and mounted as previously described [33]. For Bcl10 cells that also expressed FLAG-vE10, cells were solubilized (0.2% Triton x-100 1x PBS 0.1% Azide) and stained with a mouse IgG_1 anti-FLAG primary antibody (Sigma) and mouse IgG_1 Alexa647 secondary antibody (Invitrogen). Image acquisition utilized an Axiovert 200M microscope and a 100x plan-apochromat oil objective

(Carl Zeiss Inc., Germany), connected to a TILL-Photonics epifluorescence imaging system for POLKADOTS analysis. LSM files were exported to Adobe Photoshop for cropping and adjustment of contrast levels. Identification of cells with POLKADOTS was performed as previously described [34].

Immunoprecipitation and western blotting. D10 T cells were collected at desired time points and lysed on ice for 30 minutes using an ubiquitin-specific lysis buffer [62]. Cell lysates were pre-cleared using protein G sepharose beads (GE) and incubated with rabbit anti-GFP antibody (Rockland) overnight at 4ºC. Antibody-bound protein complexes were then captured using protein G sepharose beads. The immunoprecipitation procedure for ubiquitinated Bcl10 was done as previously described [59] with additional steps that included the addition of 1% SDS to the lysates, which were heated for 5 min at 95º C. Lysates were then diluted to a concentration of 0.1% SDS, followed by overnight immunoprecipitation. Western blotting was done as described [34].

For co-immunoprecipitation of pcDNA3 cloned 3xHA-Bcl10 and 3xFLAG-tagged partner proteins (e.g. MALT1, CARMA1, VE10, Bcl10), each sample consisted of 1.2×10^6 HEK293T cells distributed between two wells of a 6-well plate. The next day, cells were transfected by the calcium phosphate method, using construct-dependent DNA concentration for pcDNA3–3xHA-Bcl10-GFP mutants and 1.5 µg of one pcDNA3-3xFLAG-tagged construct per well. On the fourth day, cells from the two wells were harvested and pooled and 1/6 of the cells were used for whole cell lysates (WCL; direct lysis and sonication in 1× Laemmli buffer). The remaining cells were incubated in IP lysis buffer [33] for 30 min on ice. Lysates were centrifuged at 4 °C (15,000 × g) for 10 min. Supernatants were pre-cleared with 10 µl of Protein

-G-Sepharose (Amersham Biosciences) with rotation at 4 °C for 1 hr. Protein complexes were then immunoprecipitated overnight with mouse anti-FLAG antibody (M2; Sigma) at 4 °C with rotation. Immunoprecipitates were captured with 20 µl of Protein-G-Sepharose with rotation at 4° C for 1 hr, and washed 3 times in 700 µl IP lysis buffer. Samples were then boiled in 1× Laemmli buffer.

Cell lysates were separated on an 8%, 10%, or 12% SDS-PAGE gel and transferred via the electrophoretic semi-dry method to nitrocellulose for blotting. Blots were blocked using either 3% BSA in 1x TBS or 5% non-fat dry milk block. For a complete list of primary and secondary antibodies used for blotting, see the **Antibodies** section.

Antibodies. Primary antibodies used in this study included mouse anti-Bcl10 (Santa Cruz sc-5273), rabbit anti-Bcl10 (Santa Cruz sc-5611), mouse anti-GAPDH (Santa Cruz sc-32233), mouse anti polyubiquitin (Santa Cruz sc-8017), rabbit anti-Cyclin D3 (Santa Cruz sc-182 and sc-6283), rabbit anti-FLAG (Sigma F7425), and rabbit anti-HA (Santa Cruz sc-805). For western blots, horseradish peroxidase–coupled goat anti-mouse or anti-rabbit secondary antibodies from Jackson ImmunoResearch were used. For immunoprecipitation, antibodies included mouse anti-FLAG (M2, Sigma) and rabbit anti-GFP (Rockland 600-401-215). For microscopy and flow cytometry, the primary antibody used was mouse anti-FLAG (M2, Sigma) with the secondary antibody anti-mouse IgG_1 Alexa647 (Invitrogen, A21240).

References

1. Janeway CA Jr, T.P., Walport M, et al., *Immunobiology: The Immune System in Health and Disease.* 5th ed., 2001, New York: Garland Science.
2. Weiss, A. and D.R. Littman, *Signal transduction by lymphocyte antigen receptors.* Cell, 1994. **76**(2): p. 263-74.
3. Konig, R., *Interactions between MHC molecules and co-receptors of the TCR.* Curr Opin Immunol, 2002. **14**(1): p. 75-83.
4. Nel, A.E., *T-cell activation through the antigen receptor. Part 1: signaling components, signaling pathways, and signal integration at the T-cell antigen receptor synapse.* J Allergy Clin Immunol, 2002. **109**(5): p. 758-70.
5. Seder, R.A. and R. Ahmed, *Similarities and differences in CD4+ and CD8+ effector and memory T cell generation.* Nat Immunol, 2003. **4**(9): p. 835-42.
6. Ghosh, S. and M.S. Hayden, *New regulators of NF-kappaB in inflammation.* Nat Rev Immunol, 2008. **8**(11): p. 837-48.
7. Hayden, M.S. and S. Ghosh, *Signaling to NF-kappaB.* Genes Dev, 2004. **18**(18): p. 2195-224.
8. Rao, A., C. Luo, and P.G. Hogan, *Transcription factors of the NFAT family: regulation and function.* Annu Rev Immunol, 1997. **15**: p. 707-47.
9. Schulze-Luehrmann, J. and S. Ghosh, *Antigen-receptor signaling to nuclear factor kappa B.* Immunity, 2006. **25**(5): p. 701-15.
10. Grakoui, A., et al., *The immunological synapse: a molecular machine controlling T cell activation.* Science, 1999. **285**(5425): p. 221-7.
11. Monks, C.R., et al., *Three-dimensional segregation of supramolecular activation clusters in T cells.* Nature, 1998. **395**(6697): p. 82-6.
12. Bubeck Wardenburg, J., et al., *Phosphorylation of SLP-76 by the ZAP-70 protein-tyrosine kinase is required for T-cell receptor function.* J Biol Chem, 1996. **271**(33): p. 19641-4.
13. Pitcher, L.A. and N.S. van Oers, *T-cell receptor signal transmission: who gives an ITAM?* Trends Immunol, 2003. **24**(10): p. 554-60.
14. Wange, R.L., *LAT, the linker for activation of T cells: a bridge between T cell-specific and general signaling pathways.* Sci STKE, 2000. **2000**(63): p. re1.
15. Berridge, M.J., M.D. Bootman, and P. Lipp, *Calcium--a life and death signal.* Nature, 1998. **395**(6703): p. 645-8.
16. Hogan, P.G., et al., *Transcriptional regulation by calcium, calcineurin, and NFAT.* Genes Dev, 2003. **17**(18): p. 2205-32.
17. Angel, P. and M. Karin, *The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation.* Biochim Biophys Acta, 1991. **1072**(2-3): p. 129-57.
18. Su, Y., et al., *Sphingosine 1-phosphate, a novel signaling molecule, stimulates DNA binding activity of AP-1 in quiescent Swiss 3T3 fibroblasts.* J Biol Chem, 1994. **269**(23): p. 16512-7.
19. Ho, N., M. Gullberg, and T. Chatila, *Activation protein 1-dependent transcriptional activation of interleukin 2 gene by Ca2+/calmodulin kinase type IV/Gr.* J Exp Med, 1996. **184**(1): p. 101-12.

20. Macian, F., *NFAT proteins: key regulators of T-cell development and function.* Nat Rev Immunol, 2005. **5**(6): p. 472-84.
21. Lee, K.Y., et al., *PDK1 nucleates T cell receptor-induced signaling complex for NF-kappaB activation.* Science, 2005. **308**(5718): p. 114-8.
22. Li, C., M.E. Fultz, and G.L. Wright, *PKC-alpha shows variable patterns of translocation in response to different stimulatory agents.* Acta Physiol Scand, 2002. **174**(3): p. 237-46.
23. Pomerantz, J.L., E.M. Denny, and D. Baltimore, *CARD11 mediates factor-specific activation of NF-kappaB by the T cell receptor complex.* EMBO J, 2002. **21**(19): p. 5184-94.
24. Senftleben, U., et al., *Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway.* Science, 2001. **293**(5534): p. 1495-9.
25. Hayashi, K. and A. Altman, *Protein kinase C theta (PKCtheta): a key player in T cell life and death.* Pharmacol Res, 2007. **55**(6): p. 537-44.
26. Sommer, K., et al., *Phosphorylation of the CARMA1 linker controls NF-kappaB activation.* Immunity, 2005. **23**(6): p. 561-74.
27. Bertin, J., et al., *CARD11 and CARD14 are novel caspase recruitment domain (CARD)/membrane-associated guanylate kinase (MAGUK) family members that interact with BCL10 and activate NF-kappa B.* J Biol Chem, 2001. **276**(15): p. 11877-82.
28. Gaide, O., et al., *Carma1, a CARD-containing binding partner of Bcl10, induces Bcl10 phosphorylation and NF-kappaB activation.* FEBS Lett, 2001. **496**(2-3): p. 121-7.
29. McAllister-Lucas, L.M., et al., *Bimp1, a MAGUK family member linking protein kinase C activation to Bcl10-mediated NF-kappaB induction.* J Biol Chem, 2001. **276**(33): p. 30589-97.
30. Lucas, P.C., et al., *Bcl10 and MALT1, independent targets of chromosomal translocation in malt lymphoma, cooperate in a novel NF-kappa B signaling pathway.* J Biol Chem, 2001. **276**(22): p. 19012-9.
31. Uren, A.G., et al., *Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma.* Mol Cell, 2000. **6**(4): p. 961-7.
32. Wegener, E. and D. Krappmann, *CARD-Bcl10-Malt1 signalosomes: missing link to NF-kappaB.* Sci STKE, 2007. **2007**(384): p. pe21.
33. Schaefer, B.C., et al., *Complex and dynamic redistribution of NF-kappaB signaling intermediates in response to T cell receptor stimulation.* Proc Natl Acad Sci U S A, 2004. **101**(4): p. 1004-9.
34. Rossman, J.S., et al., *POLKADOTS are foci of functional interactions in T-Cell receptor-mediated signaling to NF-kappaB.* Mol Biol Cell, 2006. **17**(5): p. 2166-76.
35. Ruefli-Brasse, A.A., D.M. French, and V.M. Dixit, *Regulation of NF-kappaB-dependent lymphocyte activation and development by paracaspase.* Science, 2003. **302**(5650): p. 1581-4.
36. Thome, M., et al., *Antigen receptor signaling to NF-kappaB via CARMA1, BCL10, and MALT1.* Cold Spring Harb Perspect Biol, 2010. **2**(9): p. a003004.
37. Rebeaud, F., et al., *The proteolytic activity of the paracaspase MALT1 is key in T cell activation.* Nat Immunol, 2008. **9**(3): p. 272-81.
38. Noels, H., et al., *A Novel TRAF6 binding site in MALT1 defines distinct mechanisms of NF-kappaB activation by API2middle dotMALT1 fusions.* J Biol Chem, 2007. **282**(14): p. 10180-9.

39. Sun, L., et al., *The TRAF6 ubiquitin ligase and TAK1 kinase mediate IKK activation by BCL10 and MALT1 in T lymphocytes.* Mol Cell, 2004. **14**(3): p. 289-301.
40. Mercurio, F., et al., *IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation.* Science, 1997. **278**(5339): p. 860-6.
41. Shambharkar, P.B., et al., *Phosphorylation and ubiquitination of the IkappaB kinase complex by two distinct signaling pathways.* EMBO J, 2007. **26**(7): p. 1794-805.
42. DiDonato, J., et al., *Mapping of the inducible IkappaB phosphorylation sites that signal its ubiquitination and degradation.* Mol Cell Biol, 1996. **16**(4): p. 1295-304.
43. Joyce, D., et al., *NF-kappaB and cell-cycle regulation: the cyclin connection.* Cytokine Growth Factor Rev, 2001. **12**(1): p. 73-90.
44. Brown, K., et al., *Mutual regulation of the transcriptional activator NF-kappa B and its inhibitor, I kappa B-alpha.* Proc Natl Acad Sci U S A, 1993. **90**(6): p. 2532-6.
45. Sun, S.C., et al., *NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway.* Science, 1993. **259**(5103): p. 1912-5.
46. Bouchier-Hayes, L. and S.J. Martin, *CARD games in apoptosis and immunity.* EMBO Rep, 2002. **3**(7): p. 616-21.
47. Hofmann, K., P. Bucher, and J. Tschopp, *The CARD domain: a new apoptotic signalling motif.* Trends Biochem Sci, 1997. **22**(5): p. 155-6.
48. McAllister-Lucas, L.M., et al., *The CARMA3-Bcl10-MALT1 signalosome promotes angiotensin II-dependent vascular inflammation and atherogenesis.* J Biol Chem. **285**(34): p. 25880-4.
49. Wang, L., et al., *Card10 is a novel caspase recruitment domain/membrane-associated guanylate kinase family member that interacts with BCL10 and activates NF-kappa B.* J Biol Chem, 2001. **276**(24): p. 21405-9.
50. Akagi, T., et al., *A novel gene, MALT1 at 18q21, is involved in t(11;18) (q21;q21) found in low-grade B-cell lymphoma of mucosa-associated lymphoid tissue.* Oncogene, 1999. **18**(42): p. 5785-94.
51. Hailfinger, S., et al., *Essential role of MALT1 protease activity in activated B cell-like diffuse large B-cell lymphoma.* Proc Natl Acad Sci U S A, 2009. **106**(47): p. 19946-51.
52. Willis, T.G., et al., *Bcl10 is involved in t(1;14)(p22;q32) of MALT B cell lymphoma and mutated in multiple tumor types.* Cell, 1999. **96**(1): p. 35-45.
53. Ruland, J., et al., *Bcl10 is a positive regulator of antigen receptor-induced activation of NF-kappaB and neural tube closure.* Cell, 2001. **104**(1): p. 33-42.
54. Langel, F.D., et al., *Multiple protein domains mediate interaction between Bcl10 and MALT1.* J Biol Chem, 2008. **283**(47): p. 32419-31.
55. Thome, M. and R. Weil, *Post-translational modifications regulate distinct functions of CARMA1 and BCL10.* Trends Immunol, 2007. **28**(6): p. 281-8.
56. Rueda, D., et al., *Bcl10 controls TCR- and FcgammaR-induced actin polymerization.* J Immunol, 2007. **178**(7): p. 4373-84.
57. Ruefli-Brasse, A.A., et al., *Rip2 participates in Bcl10 signaling and T-cell receptor-mediated NF-kappaB activation.* J Biol Chem, 2004. **279**(2): p. 1570-4.
58. Ishiguro, K., et al., *Ca2+/calmodulin-dependent protein kinase II is a modulator of CARMA1-mediated NF-kappaB activation.* Mol Cell Biol, 2006. **26**(14): p. 5497-508.
59. Wu, C.J. and J.D. Ashwell, *NEMO recognition of ubiquitinated Bcl10 is required for T cell receptor-mediated NF-kappaB activation.* Proc Natl Acad Sci U S A, 2008. **105**(8): p. 3023-8.

60. Lobry, C., et al., *Negative feedback loop in T cell activation through IkappaB kinase-induced phosphorylation and degradation of Bcl10.* Proc Natl Acad Sci U S A, 2007. **104**(3): p. 908-13.
61. Paul, S., et al., *Selective Autophagy of the Adaptor Protein Bcl10 Modulates T Cell Receptor Activation of NF-kappaB.* Immunity,, (1097-4180 (Electronic)).
62. Scharschmidt, E., et al., *Degradation of Bcl10 induced by T-cell activation negatively regulates NF-kappa B signaling.* Mol Cell Biol, 2004. **24**(9): p. 3860-73.
63. Gao C, et al., *Autophagy negatively regulates Wnt signalling by promoting Dishevelled degradation.* Nat, Immunol, (1476-4679 (Electronic)).
64. Shi, C., et al., *Activation of autophagy by inflammatory signals limits IL-1beta production by targeting ubiquitinated inflammasomes for destruction.* Nat, Immunol, (1529-2916 (Electronic)).
65. Sixt, M., et al., *The conduit system transports soluble antigens from the afferent lymph to resident dendritic cells in the T cell area of the lymph node.* Immunity, 2005. **22**(1): p. 19-29.
66. Shen, L., et al., *Aberrant BCL10 nuclear expression in nasal NK/T-cell lymphoma.* Blood, 2003. **102**(4): p. 1553-4.
67. Ye, H., et al., *BCL10 gene amplification associated with strong nuclear BCL10 expression in a diffuse large B cell lymphoma with IGH-BCL2 fusion.* Haematologica, 2006. **91**(6 Suppl): p. ECR28.
68. Yeh, P.Y., et al., *A pathway for tumor necrosis factor-alpha-induced Bcl10 nuclear translocation. Bcl10 is up-regulated by NF-kappaB and phosphorylated by Akt1 and then complexes with Bcl3 to enter the nucleus.* J Biol Chem, 2006. **281**(1): p. 167-75.
69. Gaide, O., et al., *CARMA1 is a critical lipid raft-associated regulator of TCR-induced NF-kappa B activation.* Nat Immunol, 2002. **3**(9): p. 836-43.
70. Nakagawa, M., et al., *MALT1 contains nuclear export signals and regulates cytoplasmic localization of BCL10.* Blood, 2005. **106**(13): p. 4210-6.
71. Kuo, S.H., et al., *Nuclear expression of BCL10 or nuclear factor kappa B predicts Helicobacter pylori-independent status of early-stage, high-grade gastric mucosa-associated lymphoid tissue lymphomas.* J Clin Oncol, 2004. **22**(17): p. 3491-7.
72. Chen, M., L.Y. Li, and Y.P. Qi, *Bcl10 protein can act as a transcription activator in yeast.* Mol Cell Biochem, 2003. **246**(1-2): p. 97-103.
73. Liu, Y., et al., *BCL10 mediates lipopolysaccharide/toll-like receptor-4 signaling through interaction with Pellino2.* J Biol Chem, 2004. **279**(36): p. 37436-44.
74. Franco, R., et al., *Nuclear bcl10 expression characterizes a group of ocular adnexa MALT lymphomas with shorter failure-free survival.* Mod Pathol, 2006. **19**(8): p. 1055-67.
75. Gatumu, M.K., et al., *Nuclear BCL10 in primary Sjogren's syndrome.* J Oral Pathol Med, 2009. **38**(6): p. 501-7.
76. Blakeslee, J.R., Jr., et al., *Evidence of respiratory tract infection induced by equine herpesvirus, type 2, in the horse.* Can J Microbiol, 1975. **21**(12): p. 1940-6.
77. Thome, M., et al., *Equine herpesvirus-2 E10 gene product, but not its cellular homologue, activates NF-kappaB transcription factor and c-Jun N-terminal kinase.* J Biol Chem, 1999. **274**(15): p. 9962-8.

78. Koseki, T., et al., *CIPER, a novel NF kappaB-activating protein containing a caspase recruitment domain with homology to Herpesvirus-2 protein E10.* J Biol Chem, 1999. **274**(15): p. 9955-61.
79. Srinivasula, S.M., et al., *CLAP, a novel caspase recruitment domain-containing protein in the tumor necrosis factor receptor pathway, regulates NF-kappaB activation and apoptosis.* J Biol Chem, 1999. **274**(25): p. 17946-54.
80. Thome, M., et al., *Equine herpesvirus protein E10 induces membrane recruitment and phosphorylation of its cellular homologue, bcl-10.* J Cell Biol, 2001. **152**(5): p. 1115-22.
81. Ho, L., et al., *MALT1 and the API2-MALT1 fusion act between CD40 and IKK and confer NF-kappa B-dependent proliferative advantage and resistance against FAS-induced cell death in B cells.* Blood, 2005. **105**(7): p. 2891-9.
82. Zhang, Q., et al., *Inactivating mutations and overexpression of BCL10, a caspase recruitment domain-containing gene, in MALT lymphoma with t(1;14)(p22;q32).* Nat Genet, 1999. **22**(1): p. 63-8.
83. Song, Z. and H. Steller, *Death by design: mechanism and control of apoptosis.* Trends Cell Biol, 1999. **9**(12): p. M49-52.
84. Zong, W.X., et al., *The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-kappaB that blocks TNFalpha-induced apoptosis.* Genes Dev, 1999. **13**(4): p. 382-7.
85. Feng, B., et al., *NF-kappaB inducible genes BCL-X and cyclin E promote immature B-cell proliferation and survival.* Cell Immunol, 2004. **232**(1-2): p. 9-20.
86. Hinz, M., et al., *NF-kappaB function in growth control: regulation of cyclin D1 expression and G0/G1-to-S-phase transition.* Mol Cell Biol, 1999. **19**(4): p. 2690-8.
87. Dong, J., et al., *Constitutively active NF-kappaB triggers systemic TNFalpha-dependent inflammation and localized TNFalpha-independent inflammatory disease.* Genes Dev. **24**(16): p. 1709-17.
88. Karin, M. and A. Lin, *NF-kappaB at the crossroads of life and death.* Nat Immunol, 2002. **3**(3): p. 221-7.
89. Alison, M.R., L.J. Nicholson, and W.R. Lin, *Chronic inflammation and hepatocellular carcinoma.* Recent Results Cancer Res, 2011. **185**: p. 135-48.
90. Papa, S., et al., *Mechanisms of liver disease: cross-talk between the NF-kappaB and JNK pathways.* Biol Chem, 2009. **390**(10): p. 965-76.
91. Jani, T.S., et al., *Inhibition of NF-kappaB signaling by quinacrine is cytotoxic to human colon carcinoma cell lines and is synergistic in combination with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or oxaliplatin.* J Biol Chem. **285**(25): p. 19162-72.
92. Kool, M., et al., *Patient and spouse appraisals of health status in rheumatoid arthritis and fibromyalgia: discrepancies and associations with invalidation.* Clin Exp, Rheumatol, (0392-856X (Print)).
93. Matmati, M., et al., *A20 (TNFAIP3) deficiency in myeloid cells triggers erosive polyarthritis resembling rheumatoid arthritis.* Nat Genet. **43**(9): p. 908-12.
94. Hayden, M.S. and S. Ghosh, *NF-kappaB, the first quarter-century: remarkable progress and outstanding questions.* Genes Dev, 2012. **26**(3): p. 203-34.
95. Lee, Y.C., et al., *Insulin-like growth factor-binding protein-3 (IGFBP-3) blocks the effects of asthma by negatively regulating NF-kappaB signaling through IGFBP-3R-mediated activation of caspases.* J Biol Chem. **286**(20): p. 17898-909.

96. Sheller, J.R., et al., *Nuclear factor kappa B induction in airway epithelium increases lung inflammation in allergen-challenged mice.* Exp Lung Res, 2009. **35**(10): p. 883-95.
97. Kingeter, L.M., et al., *Cutting edge: TCR ligation triggers digital activation of NF-kappaB.* J Immunol, 2010. **185**(8): p. 4520-4.
98. Hayden, M.S., A.P. West, and S. Ghosh, *NF-kappaB and the immune response.* Oncogene, 2006. **25**(51): p. 6758-80.
99. Hayden, M.S., A.P. West, and S. Ghosh, *SnapShot: NF-kappaB signaling pathways.* Cell, 2006. **127**(6): p. 1286-7.
100. Snapper, C.M., et al., *B cells from p50/NF-kappa B knockout mice have selective defects in proliferation, differentiation, germ-line CH transcription, and Ig class switching.* J Immunol, 1996. **156**(1): p. 183-91.
101. Ruland, J., et al., *Differential requirement for Malt1 in T and B cell antigen receptor signaling.* Immunity, 2003. **19**(5): p. 749-58.
102. Sun, Z., et al., *PKC-theta is required for TCR-induced NF-kappaB activation in mature but not immature T lymphocytes.* Nature, 2000. **404**(6776): p. 402-7.
103. Hayashi, Y., et al., *BCL10 single nucleotide polymorphism and its association with CSF dissemination of primary intracranial germ cell tumors.* Pediatr Neurosurg, 2009. **45**(4): p. 291-5.
104. Inoue, T., et al., *Association of BCL10 germ line polymorphisms on chromosome 1p with advanced stage testicular germ cell tumor patients.* Cancer Lett, 2006. **240**(1): p. 41-7.
105. Gill, S., et al., *BCL10 is rarely mutated in human prostate carcinoma, small-cell lung cancer, head and neck tumours, renal carcinoma and sarcomas. MPT Collaborators, St George's Hospital Collaborators.* Br J Cancer, 1999. **80**(10): p. 1565-8.
106. Grimwade, D., et al., *Screening for mutations of Bcl10 in leukaemia.* Br J Haematol, 2000. **109**(3): p. 611-5.
107. Lambers, A.R., et al., *Bcl10 is not a target for frequent mutation in human carcinomas.* Br J Cancer, 1999. **80**(10): p. 1575-6.
108. Takahashi, H., et al., *Infrequent BCL10 mutations in B-cell non-Hodgkin's lymphomas.* Jpn J Cancer Res, 1999. **90**(12): p. 1316-20.
109. Moscat, J., P. Rennert, and M.T. Diaz-Meco, *PKCzeta at the crossroad of NF-kappaB and Jak1/Stat6 signaling pathways.* Cell Death Differ, 2006. **13**(5): p. 702-11.
110. Seibenhener, M., et al., *Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation.* Mol Cell, Biol, (0270-7306 (Print)).
111. Wegener, E., et al., *Essential role for IkappaB kinase beta in remodeling Carma1-Bcl10-Malt1 complexes upon T cell activation.* Mol Cell, 2006. **23**(1): p. 13-23.
112. Guiet, C. and P. Vito, *Caspase recruitment domain (CARD)-dependent cytoplasmic filaments mediate bcl10-induced NF-kappaB activation.* J Cell Biol, 2000. **148**(6): p. 1131-40.
113. Schaefer, B., et al., *A novel family of retroviral vectors for the rapid production of complex stable cell lines.* Anal, Biochem, (0003-2697 (Print)).
114. Jordan M., Schallhorn A., and F.M. Wurm, *Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation.* 1996(0305-1048 (Print)).
115. Kaye, P., A. Curry, and J.M. Blackwell, *Differential production of Th1- and Th2-derived cytokines does not determine the genetically controlled or vaccine-induced rate of cure in murine visceral leishmaniasis.* J. Immunol, 1991(0022-1767 (Print)).

116. Lobry, C. and R. Weil, *[New Bcl10 regulation mechanisms: a step in the comprehension of what has occurred in MALT lymphomas?].* Med Sci (Paris), 2007. **23**(4): p. 353-5.
117. Guelen, L., et al., *TAT-apoptin is efficiently delivered and induces apoptosis in cancer cells.* Oncogene,, (0950-9232 (Print)).
118. Klionsky, D.J., et al., *Does bafilomycin A block the fusion of autophagosomes with lysosomes?* Autophagy, 2008. **4**(7): p. 849-850.
119. Ishiguro, K., et al., *Bcl10 is phosphorylated on Ser138 by Ca2+/calmodulin-dependent protein kinase II.* Mol Immunol, 2007. **44**(8): p. 2095-100.
120. McAllister-Lucas, L.M., et al., *CARMA3/Bcl10/MALT1-dependent NF-kappaB activation mediates angiotensin II-responsive inflammatory signaling in nonimmune cells.* Proc Natl Acad Sci U S A, 2007. **104**(1): p. 139-44.
121. Kaye, J., et al., *Both a monoclonal antibody and antisera specific for determinants unique to individual cloned helper T cell lines can substitute for antigen and antigen-presenting cells in the activation of T cells.* J Exp Med, 1983. **158**(3): p. 836-56.
122. Haughton, G., et al., *The CH series of murine B cell lymphomas: neoplastic analogues of Ly-1+ normal B cells.* Immunol Rev, 1986. **93**: p. 35-51.
123. Schaefer, B.C., et al., *Live cell fluorescence imaging of T cell MEKK2: redistribution and activation in response to antigen stimulation of the T cell receptor.* Immunity, 1999. **11**(4): p. 411-21.